The Crossroads of Tissue Growth and Metabolism in Liver Regeneration

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1. Summary

The capacity of liver to regenerate has enabled the surgical removal of large liver parts for the treatment of malignancy. Surgery offers the best chance for a cure from liver cancer; due to the ongoing shortage in donor organs limiting transplantation, hepatectomy has become the most frequent intervention against disease. However, the regenerative capacity of liver is not endless. While up to two thirds of liver can be safely removed, larger resections bear the risk of liver failure and subsequent death. Resection-induced liver failure (also known as the Small-for-Size Syndrome, SFSS) represents a major constraint for the application of surgery. The pathobiology behind the SFSS is poorly understood. Therefore, knowledge about the fundamental principles that limit the regenerative capacity of liver is needed to develop novel strategies against the SFSS.

Liver is the central metabolic organ vital for the body. Following tissue loss, liver not only needs to recover mass, but must maintain essential metabolic tasks. Therefore, metabolic function needs to be coordinated with tissue growth during regeneration. Moreover, metabolism has to be adapted to provide energy for regeneration, a process that is ill-understood hitherto. SFSS liver illustrates both the growth-related and metabolic aspects of liver regeneration; here, liver failure is associated with a deficient progression through the hepatocyte cell cycle and with metabolic deficits, such as hyperbilirubinemia or persisting steatosis.

Intriguingly, persisting steatosis is generally observed in situations of incomplete recovery after hepatectomy. Indeed, every regenerating liver transiently accumulates lipids early after tissue loss. If regeneration is successful, this regeneration-associated steatosis (RAS) vanishes with the major wave of parenchymal growth. If regeneration however fails, RAS persists such as in the SFSS. While RAS meanwhile is considered an essential component of regeneration, its function remains to be clarified.

One general aim of my PhD thesis was to explore the function of RAS. The other overall aim was to get insight into the reciprocal control that aligns growth with the metabolic performance of liver.

With regards to RAS, I investigated the regenerative role of PTEN, the well-known tumor suppressor which regulates growth and metabolism through the inhibition of the AKT-mTOR axis. In wild type mice, we observed PTEN downregulation at the peak of RAS after standard (68%) hepatectomy. Using pharmacological modulation and inducible, hepatocyte-specific Pten-/- mice, we identified PTEN downregulation as a promoter of cellular hypertrophy leading to a gain in functional liver mass. Moreover, PTEN deficiency inhibited glucose usage but enhanced the oxidation of lipids. Mild inhibition of β-oxidation led to the persistence of RAS and the suppression of hypertrophic growth, with the latter being dependent on mTOR activities. These findings indicate that PTEN downregulation

promotes the burning of RAS-derived lipids to fuel hypertrophic regeneration after hepatectomy. Therefore, one key function of RAS is to provide energy for the regenerative process.

The role of PTEN in liver regeneration additionally highlights mechanisms that regulate tissue growth through the adaptation of energy metabolism. To obtain further understanding of the association between tissue growth and the maintenance of metabolic liver function, we investigated the contribution of the constitutive androstane receptor (CAR) to the regenerative process. CAR is a xeno/endobiotic sensor that upon activation induces the transcription of genes required for substance detoxification/metabolism. Intriguingly, strong CAR activation causes spontaneous hepatomegaly in mice, suggesting a coupling of CAR's metabolic function to regenerative capacities. Using Car-/- mice, CAR activators and knockdown approaches, we demonstrated that CAR activation is required to push hepatocytes through mitosis via the induction of the cell cycle molecule FOXM1. If the CAR-FOXM1 axis fails, mice are prone to liver failure after hepatectomy and display all typical SFSS features. Importantly, we observed failed induction of CAR also in human SFSS liver, with CAR stimulation promoting proliferation in ex vivo human liver slices. Finally, exogenous CAR activation rescued mice from lethal liver failure and normalized the SFSS phenotype in a way dependent on FOXM1. The normalization of metabolic deficiencies via a cell cycle molecule illustrates the requirement for sufficient liver mass to maintain metabolic function.

In conclusion, PTEN and CAR function in liver regeneration to coordinate tissue growth with energy metabolism and metabolic capacity, respectively. While metabolism feeds liver growth via PTEN, liver growth feeds metabolism via CAR. Importantly, both the inhibition of PTEN and the activation of CAR are able to rescue mice from lethal forms of resection-induced liver failure. Pharmacological modulation of these proteins might hence provide opportunities to mitigate the SFSS in the clinic. No treatment currently exists for the SFSS, and his entity remains the leading cause of death due to liver surgery.

2. Zusammenfassung

Die Fähigkeit der Leber, nach Gewebeverlust zu regenerieren, hat chirurgische Eingriffe bei grossen Tumoren ermöglicht. Die Chirurgie eröffnet die besten Chancen für eine kurative Therapie eines Leberkrebses. Der sich verschärfende Organmangel limitiert die Anwendung der Transplantation, sodass die Resektion die häufigste chirurgische Intervention bei malignen Erkrankungen darstellt. Die regenerative Kapazität der Leber ist jedoch begrenzt. Während eine Resektion von zwei Dritteln der Leber toleriert wird, ist die Entfernung von grösseren Teilen des Organs mit der Gefahr eines möglicherweise letalen Leberversagens verbunden. Dieses Leberversagen, auch 'Small-for-Size Syndrome' (SFSS) genannt, ist eine ernstzunehmende Komplikation und muss bei der Planung des Eingriffes berücksichtigt werden. Die Pathophysiologie des SFSS ist ungeklärt. Deshalb ist ein umfassendes Verständnis der Vorgänge, welche die Leberregeneration fördern oder hemmen, notwendig, um Strategien zur Vermeidung dieses Syndroms zu entwickeln.

Die Leber ist das zentrale metabolische Organ und übernimmt dabei lebenswichtige Funktionen. Nach Gewebeverlust muss die Leber nicht nur nachwachsen, sondern auch essentielle metabolische Funktionen aufrechterhalten. Deshalb müssen Wachstum und metabolische Funktionen während der Regeneration koordiniert werden. Zudem muss der Metabolismus so adaptiert werden, dass genügend Energie für die Regeneration zur Verfügung gestellt wird. Dieser Prozess ist bis anhin kaum verstanden. Die durch SFSS beeinträchtigte Leber illustriert sowohl die Wachstums-abhängigen wie auch die metabolischen Grenzen der Leberregeneration; einerseits ist Leberversagen charakterisiert durch eine verhinderte Zellteilung, andererseits durch metabolische Defizite, wie z.B. Hyperbilirubinemie und persistierende Steatose.

Interessanterweise wird persistierende Steatose bei allen Formen einer Leberregenerationstörung beobachtet. In der Tat, jede regenerierende Leber durchläuft eine Steatose, welche sich vor der maximalen Wachstumswelle wieder auflöst. Kann diese Wachstumswelle nicht abgeschlossen werden - wie bei SFSS - bleibt die Steatose bestehen. Mittlerweile ist klar, dass diese Regenerations-assozierte Steatose (RAS) notwendig für den Lebernachwuchs ist, aber deren genaue Funktion noch unverstanden ist.

Das Ziel dieser Arbeit war, einerseits die Funktion von RAS zu erforschen, und andererseits die Koordination von Wachstum und Metabolismus während der Leberregeneration besser zu verstehen.

In Bezug auf RAS haben wir die Rolle von PTEN, einem bekannten Tumorsuppressor, untersucht, welcher Wachstum und Metabolismus durch die Inhibition des Akt-mTOR Signalweges reguliert. Nach einer Standardhepatektomie (68%) haben wir in Wildtyp-Mäusen eine Reduktion von PTEN während der RAS-Phase beobachtet. Mittels pharmakologischer Eingriffe und genetisch modifizierter Mäuse,

bei welchen Pten induziert nur in Hepatocyten ausgeschaltet werden kann, konnten wir nachweisen, dass die Reduktion von PTEN zu einer hypertrophen Reaktion mit Zunahme von funktioneller Lebermasse führt. Weiter reduzierte sich der Verbrauch von Glucose, während die Verbrennung von Fetten anstieg. Eine milde, pharmakologische Inhibition der -Oxidation führte zu einer verlängerten RAS-Phase begleitet durch eine Reduktion im hypertrophen Wachstum, welches abhängig von mTOR war. Diese Experimente zeigen, dass eine Reduktion von PTEN zur Metabolisierung der akkumulierten RAS-Lipide führt und damit das Wachstum nach Resektion fördert. Demzufolge ist RAS eine wichtige Energiequelle, welche notwendig für eine erfolgreiche Regeneration der Leber ist.

Die Rolle von PTEN in der Leberregeneration illustriert Mechanismen, welche Gewebewachstum durch eine Adaption des energetischen Metabolismus fördern. Andererseits muss auch die metabolische Funktion der regenerierenden Leber aufrechterhalten werden. Um dies besser zu verstehen, haben wir die regenerative Rolle des 'Constitutive Androstane Receptor' (CAR) untersucht. CAR ist ein Sensor für Xeno/Endobiotika und induziert Gene, welche für die Metabolisierung verschiedenster Substanzen benötigt werden. Interessanterweise induziert der voll-aktivierte CAR eine Hepatomegalie, d.h. er regt das Wachstum der Leber in der Abwesenheit von jeglichem Gewebeverlust an. Das impliziert eine Koppelung von Wachstum und Metabolismus. Mittels CAR-defizienten Mäusen (Car-/-), CAR Aktivatoren und mRNA Interferenz Experimenten konnten wir zeigen, dass CAR für das Abwickeln des vollständigen Zellzyklusprogramms notwendig ist. Nach einer Hepatektomie aktiviert CAR dabei die Mitose durch ein wichtiges Zellzyklusmolekül genannt FOXM1. Wenn die CAR-FOXM1 Achse anch einer Hepatektomie nicht induziert wird, entsteht ein Leberversagen mitsamt den klassischen SFSS Symptomen.

Auch in der menschlichen Leber von SFSS Patienten beobachteten wir eine fehlende CAR Aktivierung, und konnten die proliferative Funktion von CAR in menschlichen ex vivo Leberschnittkulturen nachweisen. Schlussendlich konnten wir zeigen, dass eine pharmakologische CAR Aktivierung Mäuse von tödlichem Leberversagen retten kann, und zwar wiederum in einer FOXM1-abhängigen Weise. Diese Erkenntnisse unterstreichen, dass eine Wiederherstellung der zentralen metabolischen Funktion der Leber auf genügend Lebermasse angewiesen ist.

Zusammenfassend zeigt diese Arbeit, dass PTEN und CAR Schnittstellen sind, welche das Leberwachstum mit dem Energiemetabolismus respektive der metabolischen Leberfunktion während der Leberregeneration koordinieren. Währen der Metabolismus das Leberwachstum via PTEN fördert, unterstützt das Wachstum die metabolische Leberfunktion via CAR. Interessanterweise kann sowohl die Inhibition von PTEN als auch die Aktivierung von CAR Mäuse vor tödlichen Formen des SFSS schützen. Pharmakologische Beeinflussung dieser Proteine könnte daher in der klinischen Situation

Abhilfe für ein sich präsentierendes SFSS schaffen. Bis anhin gibt es keine Behandlung dieses Syndroms, welches nach wie vor die häufigste Todesursache nach einer Leberoperation darstellt.

3. Introduction

3.1 Liver

The liver is both: the largest internal organ and gland in the human body. Located in the upper abdominal cavity, it carries out a wide range of vital functions. Two main vessels supply the liver with blood; the hepatic artery supplies oxygenated blood, while the portal vein provides mesenteric blood drained from the entire gastrointestinal tract including spleen and pancreas, thus blood rich in toxins/nutrients but poor in oxygen. The branches of the portal vein, the hepatic artery unite with hepatic bile ducts to form structures known as portal tracts, which permeate the entire liver tissue. De-oxygenated blood flows into central vein of each lobule. The central veins converge into hepatic veins, which finally drain into the inferior vena cava. The unique vascularization is required for liver to perform its tasks, the clearance of harmful metabolites and the production of digestive bile. Moreover, liver produces proteins for blood plasma, hormones and cholesterol, participates in immune responses and contributes to blood coagulation. Finally, liver stores glycogen, is the main provider of bodily glucose, and is able to synthesize fatty acids, triglycerides and lipoproteins for transport across the body. In simple words, liver is the metabolic center of the body.

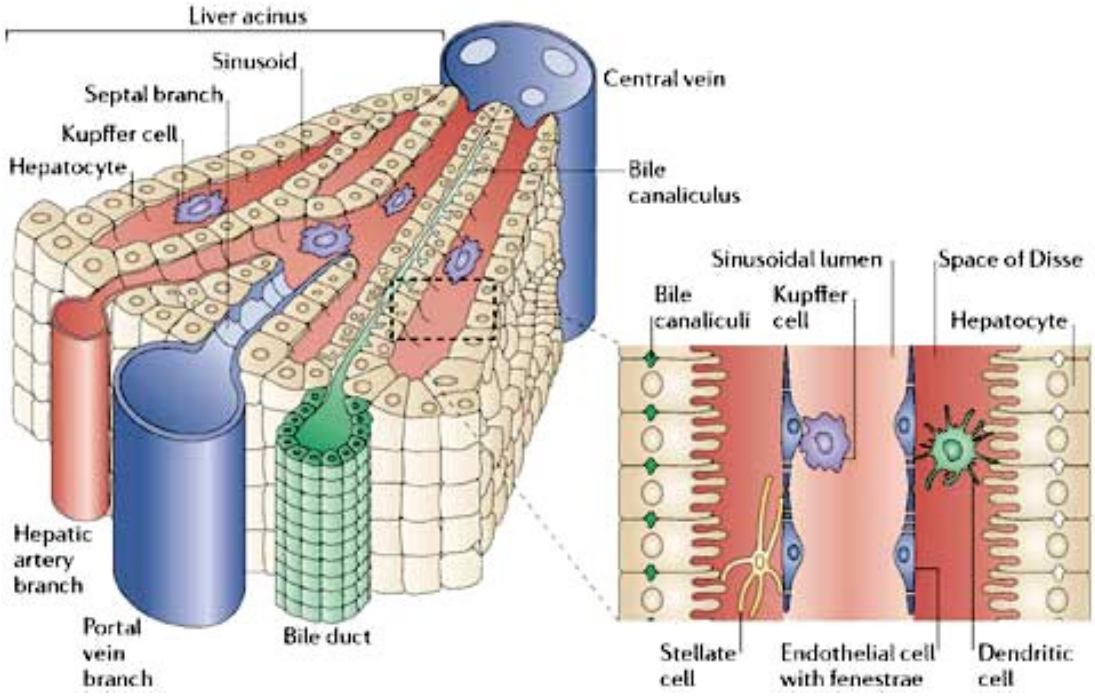

Figure1. Structure of liver acinus and sinusoid. Adapted from [1].

Liver parenchyma is the main contributor to hepatic mass and consists of highly differentiated epithelial cells, the hepatocytes [HEPs]. HEPs are closely associated with the hepatic blood flow, with each HEP lining a liver sinusoidal endothelial cell [LSECs]. LSECs form the sinusoids, which further are populated with liver-resident macrophages, the phagocytic Kupffer cells. Blood flows into sinusoids

from the terminal branches of the portal vein and hepatic artery, and then is drained into the terminal hepatic veins. The Space of Disse is defined as the interface between LSECs and HEPs. It is rich in extracellular matrix and hosts stellate cells as well as dendritic cells. As a special feature, LSECs have fenestrated membranes, enabling efficient exchange between HEPs and blood, a key requirement for proper liver function. Altogether, the sinusoid and its surrounding cells form the hepatic acinus - the functional unit of the liver (Fig.1).

3.2 Liver regeneration

The vital function of liver renders it indispensable for life. Moreover, the chronic exposure of liver to toxins has its toll. The liver hence is equipped with a unique ability to regenerate, ensuring lasting function throughout the organismal life span. Rapid recovery until original volume happens. Following significant tissue loss (up to 70% of liver volume), liver regains its original volume within 2-3 weeks in human, and about one week in mouse. The standard mouse model for the study of liver regeneration is 70% partial hepatectomy [sHx]. First presented in 1931 by Higgins&Anderson, sHx takes advantage of the multilobular structure of rodent liver and enables tissue removal without introducing injury that may cause inflammation and necrosis [2]. Besides resection, toxic liver damage also can trigger a regenerative response. However, regeneration of an injured liver occurs different from sHx, in that it strongly depends on the regrowth from stem cells [3]. Recovery of liver from toxic insults will hence not be covered here.

General principles of liver regeneration

Following sHx, liver regenerates via both enlargement and synchronized division of differentiated hepatocytes. The major mitotic wave in mice is observed around 48 hours after sHx. The regenerative process is tightly regulated, and the changes that occur within the first 2 days after liver resection are decisive for a successful completion. LSECs, the largest non-parenchymal population, enter proliferation after the major parenchymal growth phase, while the repopulation of other non-parenchymal cells is less defined. Notably, during these first two days, liver accumulates significant amounts of fat, which are thought to originate from peripheral adipose stores and vanish again around the peak of parenchymal growth. This transient lipid accumulation meanwhile is recognized as an obligate component required for successful regeneration. Although its function is unclear, lipids conceivably might serve to fulfill the demands of a growing tissue for energy and building material.

Despite the fact that liver regeneration [LR] has been extensively studied in the past, knowledge about universal signals launching the regenerative response upon resection is missing. Likewise, how liver manages to restore its volume while proceeding with metabolic activity remains unresolved. Finally, the termination process is poorly understood.

However, a pleiad of mechanisms crucial for the entry of hepatocytes into and their further progression through the cell cycle has been identified. Together, these observations portray a picture where the precise orchestration of molecular and biochemical events - rather than the activation of individual pathways - enables rapid recovery of functional liver weight following resection.

Liver regeneration and its different phases

In general, LR comprises several phases that may be timely categorized as follows: 1) the *priming phase* includes early events that prepare HEPs to enter into cycle; 2) during the *initiation and progression* phase, HEPs enter the cell cycle and proceed through mitosis; 3) the subsequent *angiogenic phase* is defined by the reconstitution of LSECs to the revascularize the rapidly growing tissue; and 4) the *termination phase,* which starts with the angiogenic phase and basically ends with the re-establishment of the original liver volume [4-6].

Priming phase. It is a common belief that 70% resection causes significant hemodynamic changes, which immediately affect the liver remnant and trigger the regenerative response. Because the portal blood inflow continues without change upon resection, the 30% remnant suddenly receives the triple amount of portal blood with according changes in blood pressure. On the other hand, arterial supply is diminished proportional to the volume loss; it hence has been proposed that the surplus of O_2-poor blood causes early hypoxia that then might unleash regenerative mechanisms. However, a recent study from our laboratory could not establish early hypoxia to play a role [7]. Better evidence exists for blood pressure-associated increases in shear stress, with sinusoidal KLF2 as a shear sensor that induces eNOS to mediate EGFR-dependent HEPs proliferation [8-10]. Further, LSECs provide mitogenic WNT2 to induce early β-catenin nuclear translocation in HEPs, with β-catenin activating the urokinase system in addition to its proliferative function [11-15]. Activation of urokinase (occurs within 5 min after hepatectomy and may as well be a result of sheer stress) and then matrix metalloproteinases is thought to cause matrix remodeling to enable the restructuring of liver tissue - but also to release matrix-bound HGF, thereby augmenting its local availability [16, 17]. Further contributions to HGF come from endothelial and stellate cells [18, 19]. At this time various cytokines derive from adjacent cells to HEPs (e.g Hedgehog, CXCRs [20], β-PDGF[21]). Given the enhanced inflow of portal blood, availability of serum growth factors per hepatocyte rises and this may as well affect LR (Insulin [22, 23], norepinephrine [24], serotonin [25], bile acids [26]).

Overall, the EGFR-ligands/HGF-MET are considered core drivers of parenchymal regeneration. These signaling axises were classified as a complete mitogens, meaning activation of these signals induces proliferation in a primary HEPs culture and results in liver enlargement, when applied to the intact animal [5]. Deregulation of single of these pathways following sHx leads to diminished G_1>S and G_2>M transitions, and often reduced G1 entry. More so, in the case of concurrent deficiency in both

pathways, liver fails to regenerate [27]. The other cytokines and growth factors are considered to play auxiliary roles, such as in controlling the precise timing of transcription factors essential for proliferation, or in enhancing the effects of the HGF-MET / EGF-EGFR pathways (Fig.2). Deficiency in one of these auxiliary factors usually leads to some regenerative delay, but is compensated through pathways with redundant function. Indeed, redundancy is a fundamental principle in LR, ensuring successful regeneration also under subopitmal conditions [5].

Initiation and progression. Under influence of mitogenic signals, received from adjacent cells and bloodstream multiple intracellular pathways become active (e.g. ERK1/2, NFkappaB, SMAD). Further activation of key transcription factors is ensured via several mechanisms. Thus, the activation of STAT3 crucial for G_1 entry, is triggered via EGFR-ligands/HGF-MET as well as IL6/TNFa derived from Kupffer cells (Fig.2) [28-31]. Induction of transcription factors STAT3, NFkappaB and AP-1 causes significant alterations in HEPs gene expression patterns [6]. Many of those relate to the cell cycle, particularly overexpression of cyclin D marks definite G_1 entry and is thought to be the "point of no return". Then proper G_1>S and G_2>M transitions depend on activation of diverse mitogenic pathways as well as their correct extent and timing. The majority of these converge on the transcription factor FOXM1, a key promoter of the S/M-phases and a repressor of the cell cycle inhibitor P21 in hepatocytes [32].

Hepatocyte proliferation starts in the periportal areas (zone 1) and then proceeds to the pericentral areas (zone 2&3) (Fig.2) [33]. Interestingly, although almost all HEPs enter the S-phase, 15% never complete the division [33]. Moreover, resent studies are questioning the established hyperplasia-dependent model of LR, because proliferation alone is not sufficient to recover the original liver mass. Rather, hyperplasia has been proposed as a core mechanism behind volume increase, given that a rapid increase in HEP size is observed within day 1 after hepatectomy [34, 35]. Hypertrophy might be an early compensatory response to immediately support tissue function and body homeostasis following tissue loss. Although requiring additional research, HEP enlargement seems to be mediated via the AKT/mTOR pathway, activated shortly after hepatectomy [34].

Angiogenic phase. Since LSECs are the first affected after hepatectomy, angiocrine signaling is pivotal for induction of parenchymal regeneration. Down-regulation of endothelial ANG2 leads to reduced production of TGFb1 (key inhibitor of HEPs proliferation) by LSECs, unleashing HEPs proliferation while LSECs themselves remain quiescent [36]. To launch HEPs into the cell cycle, HGF previously bound to extra-cellular matrix becomes available due to tissue remodeling. The regenerative stimulus is amplified via HGF and WNT2 produced by endothelial cells in VEGF/VEGFR1/2 –dependent manner [37]. Moreover, VEGF stimulates parenchymal proliferation also via hepatocellular VEGFR1 engagement [37]. Furthermore, VEGF released in the bloodstream recruits to the liver bone marrow derived progenitor cells of LSECs (BM SPCs) [38], that are rich in HGF [39] (Fig.2).

While growing parenchymal mass, liver develops hypoxia. HEPs induce a hypoxic response via HIF2a, which has a dual function. It promotes progression of the HEPs through the mitosis, and simultaneously induces *Vegf* gene expression, leading to the major wave of VEGF, essential for subsequent angiogenic phase [7]. Simultaneously re-elevated ANG2 upregulates TGFb1 to decline HEPs proliferation after mitotic peak [36]. Among BM SPCs, resident progenitor cells or LSECs, BM SPCs are considered to be major contributors to the restoration of sinusoidal vasculature as progenitors of LSECs [40, 41]. Complex angiogenic process starts at day 4 after hepatectomy in mice and will be completed within next 3-4 days [9]. Crosstalk between LSECs and HEPs described here is a notable example of mutual intercellular coordination, which assures highly ordered and efficient process of LR (Fig.2).

Termination. Termination of liver regeneration is the least understood phase. Upregulation of TGFb1 clearly is an important signal for HEPs to cease their proliferation. The remodeling of the extracellular matrix occurring early after sHx contributes to TGFb1 regulation as it releases ECM (extracellular matrix) bound active TGFb1 into plasma, where it gets inactivated by absorption through alpha-2-macroglobulin [42-44]. Over time, when the wave of pro-regenerative factors is declining, and when sinusoidal repopulation enables the reconstruction of a more ordered liver architecture, the ECM is being rebuilt by stellate cells. Ongoing with rebuilding, more and more of newly synthesized TGFb1 gets rebound to the ECM, re-establishing a state of hepatocellular quiescence as it occurs in resting liver [45-47]. Besides TGFb1, the reconstruction of the ECM also relocates in close proximity to HEPs decorin, which counteracts the key mitogenic pathways downstream of MET and EGFR [48-51]. Moreover, ECM ties down free HGF to inactivate it. Finally, ECM remodeling leads to upregulation of ILK and GCP3, which - through ill-defined paths likely involving Yap signaling contribute to termination of LR [52, 53].

There is evidence indicating some exaggeration of the regenerative response leading to overgrowth. Eventually, the number of HEPs is larger than in the original liver. This number is re-adjusted by a small wave of apoptosis eliminating excess HEPs. This process perhaps is regulated by dynamic contributions of MST1/2-YAP1 pathway, famous for its function in organ-size control [54, 55]. Nuclear YAP1 promotes proliferation, anti-apoptosis and “stemness” [56]. It is negatively controlled by MST1/2 kinase which targets YAP1 for degradation [57]. By day 1 after hepatectomy the activity of MST1/2 kinase is attenuated, resulting in elevated YAP1 activity and its downstream targets. When the original liver-to-body weight ratio is restored, MST1/2 activity rises back to the baseline level and stops YAP1 action [54]. In common agreement, the downregulation of YAP1 is considered to be an endpoint event in the termination of regeneration also through the induction of the apoptotic wave.

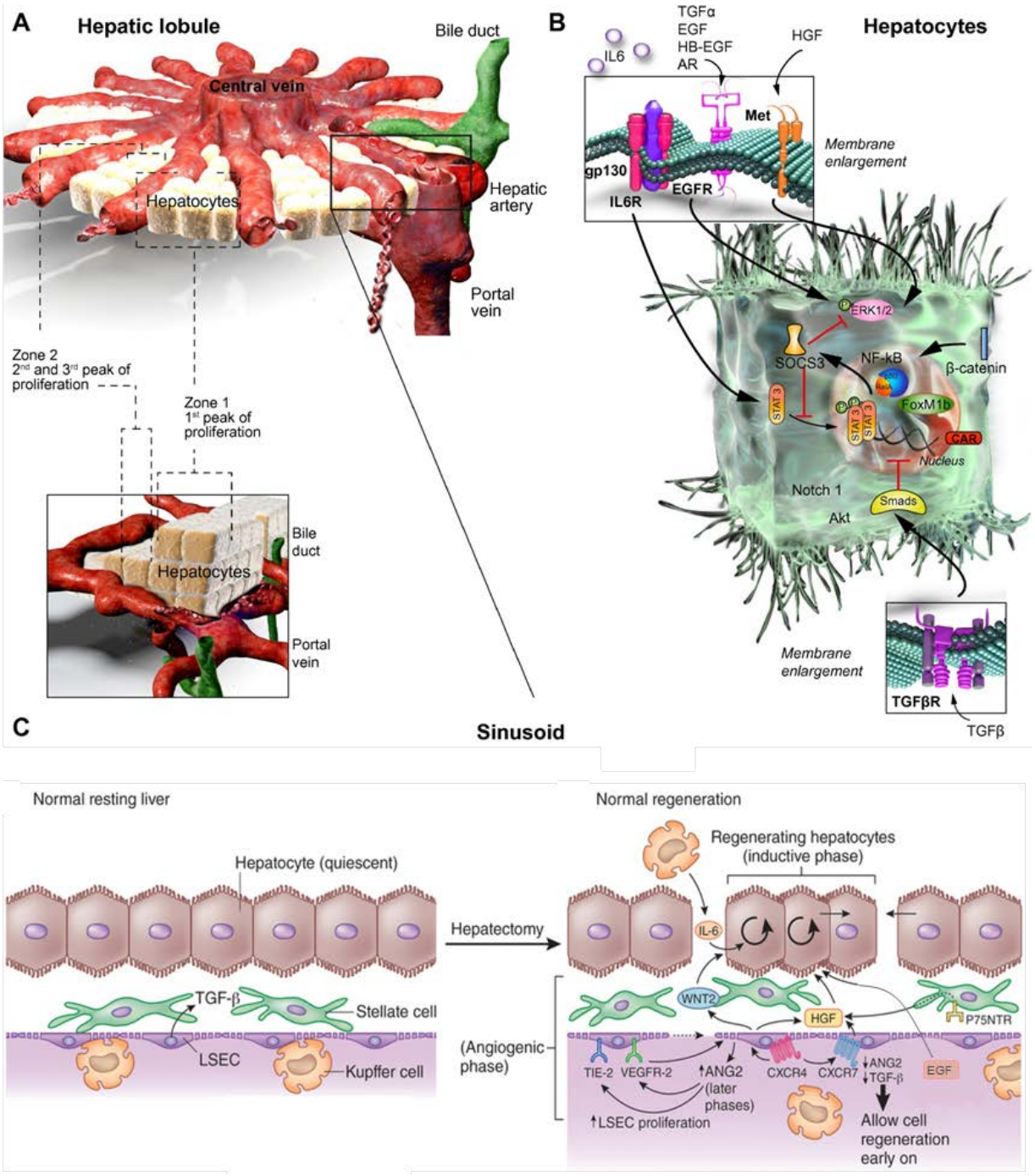

Figure 2. Liver regeneration from three different perspectives. (A) Structure of the liver lobule. Zone 1 hepatocytes (periportal; closest to portal vein) proliferate first after sHx, whereas zone 2 hepatocytes contribute to the second and third proliferative peaks. (B) Key intracellular signaling pathways in hepatocytes after PH. Adapted from [58] with modifications . (C) In the intact liver, HEPs are mitotically quiescent. LSECs secrete TGFb1, which acts as a proliferation brake on HEPs. Following sHx, LSECs downregulate ANG2 and TGFb1 during the early phase of regeneration, though ANG2 is expressed in the later angiogenic phase, during which it activates VEGFR2 and TIE2 signaling. Endothelial cells provide HGF and WNT2 and the cytokines CXCR7 and CXCR4, stellate cells - HGF and Kuppfer cells - IL6. These factors act with circulating factors, e.g EGF to stimulate HEP proliferation. Proliferation of LSECs, hepatic macrophages and stellate cells occurs later. Adapted from [20] with modifications.

However, akin to the signals that are needed for the initiation of LR, a combination of various events is likely needed for a proper termination of liver regrowth. The decline in pro-mitogenic factors will add, but signals of a broader nature are likely to contribute, such as systemic responses that sense the re-installation of normal hepatic capacity. Recent evidence indicates regulation of termination through the bodily bile acid pool that liver must circulate and which obviously will relate to the actual size of liver, reflecting the state of hepatostat [59].

3.3 Regeneration, liver surgery and the associated limits

The capacity of liver to regenerate is fundamental to liver surgery, because it enables the removal of large parts of liver such as for the treatment of liver tumors.

Small-for-Size-Syndrom

Despite major efforts in improving the management of liver tumors, surgery continues to offer the best chance for a complete cure. While the application of transplantation is restricted due to the ongoing shortage in donor organs, the resection of diseased liver parts is the most frequent intervention against liver malignancies. However, resection has its own limitations, with the most important being the extent of hepatectomy required for a complete removal of all cancerous parts. In healthy humans, up to 75% of liver mass can be removed without major risks [60]. Above this threshold, the liver remnant fails to recover. The small liver mass is unable to meet the functional demands of the body, with the developing liver failure putting patients at serious risks. In the clinic, this entity is known as Small-for-Size Syndrome [SFSS] and is the most frequent cause of death due to liver surgery. Therefore, SFSS is a serious factor limiting the application of liver surgery for the cure of malignancy.

Deficient regeneration as a cause of the SFSS

Why small liver remnants fail to recover is unclarified. It has been suggested that the massive increase in portal flow after extended hepatectomy may damage the sinusoidal lining, with persistent injury at the LSEC-HEP interface compromising liver function. In clinical studies, however, no clear association between portal pressure and the SFSS risk could be established [61, 62]. The contributions of portal pressure and liver injury to the SFSS hence are still under debate.

To better understand the pathophysiology behind resection-induced liver failure, our lab has designed a new mouse model of the SFSS. The model is based on a modified extended 86% hepatectomy [eHx] designed to protect main hepatic vessels and ducts during surgery. Although mice after eHx do not display hepatic injury as assessed through multiple parameters, they present with features typically observed in human SFSS, including persistent steatosis, a reduced metabolic liver capacity, and increased mortality [63]. These findings therefore argue against liver damage as a requirement for the

SFSS to develop.

Instead, the findings from this new SFSS model point to the simple failure of regeneration as a cause of the SFSS. Comparing the sHx and eHx models, no major differences were observed for the entry of HEPs into the cell cycle (e.g. via Ki67 and cyclin E/D levels). Following eHx, however, HEPs displayed a deficient progression through the S and particularly the M cell cycle phase, accompanied by an upregulation of the cell cycle inhibitor P21. Ablation of P21 corrected these cell cycle deficits after eHx, enhanced the liver weight regain, and improved both the metabolic SFSS features and survival [63]. Therefore, means that improve the regenerative capacity of the liver may be of clinical value with regards to the SFSS. For example, the forkhead transcription factor FOXM1 is a key promoter of the S and M phases in regenerating hepatocytes, amongst other owing to its ability to promote S/M phase cyclins and to repress P21 [32]. Indeed, our lab could demonstrate a deficient induction of the *Foxm1* gene after eHx [32, 63], perhaps suggesting a crucial role of timely FOXM1 upregulation for the outcome of extended liver resection.

Management of the SFSS

No recommended treatment currently exists for the management of the SFSS. A novel strategy relies on functional liver support employing a type of hepatic dialysis. This bio-artificial liver device [BAL] is a bioreactor consisting of HEPs that are separated from blood with a semipermeable membrane. The membrane shall mimic fenestrated LSECs, giving HEPs access to blood-derived toxins and proteins but no larger objects. In this way, HEPs can perform normal liver function tasks without provoking immunological responses [64]. Obviously, BAL could only serve as a temporary support, either extending the time window for small liver remnants to regenerate, or to take over liver function until a transplant is available.

On the other hand, strategies that aim at improving the regenerative capacity of liver might be better suited for an SFSS management. Such an approach may prevent or treat the SFSS directly, not only improving the outcome of patients undergoing extended hepatectomy, but extending the application of surgery previously deemed unresectable due to a risk of SFSS [65].

Experimental strategies in such a direction may come from the work of Katagiri et al [66, 67]. These researchers observed that a distinct fraction of bone marrow-derived mesenchymal stem cells named Muse differentiate into liver-lineage cells (HEPs, LSECs and Kupffer cells) and contribute to tissue repair [66, 67]. Thus, stem cell based therapies may aid the management of the SFSS.

Currently, the main approach is to prevent the SFSS altogether by surgical enlargement of the future liver remnant. The most important development here are the so-called two-staged hepatectomies, where in a first step healthy liver mass is enlarged (such as through the ligation of diseased liver parts, which then provokes compensatory liver growth of unligated parts). Following successful liver growth,

the second step, i.e. the resection, is then performed on the enlarged liver, leaving a remnant big enough to prevent SFSS development. However, these approaches require repeated intervention, which may affect the progression of background disease, and have a limited time window for application. Perhaps the most promising procedure here is ALPPS, a two-staged hepatectomy where the first step combines portal vein ligation with a parenchymal transection. The ligation-transection combination leads to a markedly accelerated volume gain, enabling the application of the second step within a much shorter time period [68]. Hopefully, the investigation of mechanisms underlying the ALPPS effects may identify new pharmacological targets that might enable the postoperative treatment of acute liver failure as well [69].

Importantly however, a few molecules with the potential as therapeutic SFSS candidates are already known. The basic principle behind these molecules is that they trigger spontaneous hepatomegaly in the absence of resection upon activation, suggesting they might be used for a pre/peri-operative enlargement of liver size to prevent/treat the SFSS. For example, activation of the nuclear receptors FXR and CAR does trigger hepatomegaly [26, 70]. A growing pool of evidence indicates that these and related molecules have pro-regenerative functions and could have curative potential in the settings of marginal liver remnants. These promising therapeutic targets include such molecules as LXR, PPARs, PPARG, PXR or estrogen receptors (reviewed in [71]). Intriguingly, these molecules are not cytokines or growth factors classically implicated in LR, but nuclear receptors that are deeply implicated in the coordination of proliferative responses with alterations in metabolic needs.

3.4 Metabolic control of liver regeneration

Liver regeneration can be viewed as an adaptation to metabolic insufficiencies after tissue loss. Removal of 70% of the liver results in the tripling of portal flow through the hepatic remnant. This implies a triple exposure of the remnant not only to growth factors, but also to metabolites and toxins. To sense these changes, hepatocytes are equipped with an array of nuclear receptors that become activated upon binding to endo- and xenobiotics. When active, the receptors translocate from the cytoplasm to the nucleus where they act a transcription factors to induce gene expression changes aimed at e.g. neutralizing harmful substances.

It is thought that these nuclear receptors also guard body homeostasis during LR. Intriguingly, many of these receptors appear to have a dual function: besides controlling metabolic circuits, nuclear receptors also regulate proliferative pathways, seemingly by translating metabolic insufficiency into mitotic signals.

One of the best studied nuclear receptors is the Farnesoid X Receptor (FXR). Being the primary sensor of bile acids, FXR regulates genes involved in bile acids synthesis, secretion, transportation, conjugation and detoxification. Bile acids are produced and secreted by the liver to aid the digestive

function of the intestines. 95% of the secreted bile acids are reabsorbed by the liver. Following hepatectomy, the hepatic bile acid influx proportionally increases with the lost tissue mass, quickly overloading the liver with potentially toxic bile acids. The subsequent activation of FXR triggers a metabolic response aimed at re-installing bile acid homeostasis. Simultaneously, FXR accelerates hepatocellular proliferation via feedback up-regulation of FOXM1b, the hepatic key promotor of cell cycle progression [16, 72]. In this way, liver size is being adapted to the changed metabolic conditions after tissue loss. Other nuclear receptors such as PXR, CAR and RXRa (the heterodimeric partner of most nuclear receptors) appear to function in a similar fashion, and their roles in the regulation of LR are being described. An interesting member of these nuclear receptors is CAR, known for its role in regulating xenobiotic responses and bilirubin clearance [73, 74]. Its activation can have a massive effect on liver and can lead to the spontaneous doubling of liver weight in mice [75]. Therefore, CAR is an intriguing candidate for the improvement of outcomes after extended resection.

3.5 Liver regeneration and energy metabolism

The extraordinary capacity of liver to regenerate after tissue loss requires an adequate energy supply. Moreover, liver is a key provider of glucose. Therefore, liver resection is a formidable challenge to energy homeostasis. Hepatectomy immediately causes systemic hypoglycemia and the associated depletion of hepatic glycogen stores. These profound changes appear to trigger a systemic response, that is the mobilization of adipose lipid stores and their redistribution from the periphery into the liver [76-79]. Lipogenesis seems to play little, if any, role in the formation of RAS. Indeed, FASKOL mice (liver-specific fatty acid synthase knockouts) exhibit normal RAS accumulation and LR after sHx [80].

Regeneration associated steatosis as an obligate component of liver regeneration.

As mentioned above, regeneration associated steatosis [RAS] peaks at 16h post sHx in mice, thus before the major wave of parenchymal growth. If LR proceeds normally, steatosis will decline and disappear somewhen around 48h to 72h after resection. When regeneration is impaired, RAS seems to persist, such as in SFSS liver featuring impaired HEP cell cycle progression [63]. One possibility is that RAS persists because no functional liver mass for the processing of lipids is provided as a consequence to failed regeneration. Yet experimental evidences suggest that hypoglycemia and formation of RAS are required for successful liver regeneration to occur [81-83]. For example, dextrose supplementation counteracts hypoglycemia and suppresses hepatectomy-induced LR via failed induction of FOXM1 and upregulation of P21 [83]. Vice versa, calorie restriction has the opposite effects and promotes the regenerative process [84]. These findings nicely illustrate the interconnection between metabolic parameters and transcriptional signaling regulating liver growth [83].

On the other hand, pharmacological or genetic strategies that abolish RAS accumulation have an anti-

regenerative effect and in some instances increase apoptotic rates [76, 85-87]. In support of a RAS as an obligate component for successful LR, HEPs undergo remarkable changes in their expression patterns during the first few hours after sHx. These changes include expression of "adipogenic phenotype" markers, suggesting the existence of a conserved transcriptional program leading to adipocytic transdifferentiation of hepatocytes specifically for the formation of RAS [87, 88]. However, in some models of deficient RAS formation (e.g. *L-Fabp*-null and *MTP*-IKO mice) LR appeared not to be strongly affected [80]. Perhaps RAS was not sufficiently inhibited in these models, or it was compensated for through adaptive lipogenesis [89] that was upregulated in these constitutive knockout models, possibly in analogy to the redundant signaling pathways that ensure LR under suboptimal conditions. [80, 89]. In any case, the weight of evidence clearly favors RAS as a required component of LR [76, 86, 87].

Putative functions of RAS.

If RAS indeed is required for LR, it should have a function. Anecdotal evidence has pointed to β-oxidation of lipids as the predominant ATP source in regenerating liver early after sHx, with the inhibition of β-oxidation lowering DNA synthesis [90]. Vice versa, infusion of lipids along with carnitine (facilitates β-oxidation via transfer of long-chain fatty acids across the mitochondrial membrane) accelerated the onset of HEP proliferation after sHx [91, 92]. It is hence plausible to speculate that the accumulation of lipids in HEPs after tissue loss serves to satisfy the energy demands of the growing parenchyme. Other observations support the view of RAS as an energy provider. The administration of adiponectin at hepatectomy promotes both β-oxidation and LR, while leptin has the contrary effect [87, 93]. Indeed, experimental manipulations that lead to a suppression of β-oxidation consistently inhibit LR but also cause the persistence of RAS [93-95], implying RAS provides the lipids that fuel regeneration.

Putative signaling axes that may contribute to RAS regulation and/or turnover.

Very little is known about molecular signaling cascades that are associated with RAS and its turnover. An important pathway implicated in the regulation of metabolism and cell/tissue growth is the AKT-mTOR axis. In the heart, in skeletal muscles, and in several cancers, mTOR activity has been reported to promote mitochondrial fatty acid metabolism and oxidative phosphorylation [96-100]. Kenerson and colleagues recently demonstrated that persistent activation of mTORC1 (one of the two mTOR complexes) in liver speeds up β-oxidation via upregulation of CPT1A (Carnitine palmitoyltransferase 1a - a rate-limiting enzyme for mitochondrial lipid oxidation). By promoting lipid catabolism, mTORC1 opposes the lipogenic effects of its upstream activating kinase AKT, thereby protecting liver from high fat diet-induced steatosis [101]. Notably, AKT has other metabolically active targets. AKT for example inhibits FOXO1, a transcription factor that is central to the regulation of gluconeogenesis,

glycogenolysis, and adipocyte differentiation. Notably, LR in *Akt1/2*-nil mice is deficient and accompanied by reduced glycogenesis and RAS formation; these defects can be reversed by the simultaneous deletion of FOXO1 [102]. Interestingly, AKT activity contributes to the regain in liver mass after sHx particularly through hypertrophic mechanisms [34, 103]. In more detail, PI3K - which receives regenerative signals directly from growth factor receptors - is a key upstream activator of AKT and promotes both hyperplastic and hypertrophic liver growth. Proliferation seems to be induced via direct activation of STAT3, while hypertrophy is dependent on AKT signaling, consistent with mTOR as a major regulator of cell size [34, 103]. Thus hyperplasia and hypertrophy can be dissociated at the level of PI3K, because the kinase can directly phosphorylate proteins (such as STAT3) but also phosphoinositides (most important here is the generation PtdIns(3,4,5)P3 (phosphatidylinositol (3,4,5)-trisphosphate)), which then activate PDK1, the upstream activating kinase of AKT. The phosphoinositide-dependent activation of AKT-mTOR is under negative control through PTEN, which thereby holds a powerful position in restricting all the metabolic and growth-related processes governed through AKT-mTOR. The importance of PTEN in the regulation of growth is emphasized through the fact that the protein is one of the most frequently mutated tumor suppressors in human cancers [104]. Indeed, subtle reductions in hepatic PTEN have been observed after resection, suggesting that PTEN alterations may contribute to the metabolic adaptations associated with the parenchymal growth after tissue loss.

3.6 GOAL AND AIMS OF THE PHD THESIS

In summary, the above introduction portrays liver regeneration as an adaptive response, with tissue loss causing metabolic insufficiency, which in turn triggers the recovery of functional liver mass. Insufficiencies related to the reduced metabolic clearance function of the liver may be sensed through nuclear receptors, which activate pathways to deal with the metabolic overload, and in parallel promote parenchymal growth to re-install the required metabolic capacity. On the other hand, after tissue loss hypoglycemia inevitably develops and provokes a systemic response, including the redistribution of peripheral fats into the liver. The peripheral import results in the formation of RAS, which in turn may serve to provide the fuel for the parenchymal growth. Consequently, an impaired RAS turnover should counteract the regenerative capacity of liver.

This PhD thesis was set out to explore the reciprocal regulation that coordinates metabolic pathways with parenchymal growth during liver regeneration. More specifically, the focus was on:

1) *The role of the nuclear receptor CAR in the promotion of hepatocellular cell cycle progression and its therapeutic potential in the settings of resection-induced liver failure*
2) *The role of PTEN in linking regeneration-associated steatosis with the energetic needs of growing parenchyme*

The following two paragraphs provide a more detailed background on the central two molecules investigated in this thesis, namely CAR and PTEN.

3.7 Constitutive androstane receptor

The nuclear receptor CAR (Constitutive Androstane Receptor) is a xeno/endobiotic sensor mainly expressed in liver and kidneys. Upon exposure to diverse exogenous and endogenous ligands, activated CAR dissociates from its cytoplasmic complex and translocates to the nucleus, where it binds to RXRa (its heterodimeric partner). Next, the CAR/RXRa heterodimer binds to DNA in order to activate expression of genes required for xenobiotic elimination. The list of known CAR targets includes genes encoding enzymes of the first (i.e. the cytochromes such as the well-established target *Cyp2b10*) and the second (*Ugt*, *Sult*, *Gst*) phases of xenobiotic metabolism [73, 105, 106]. Likewise, CAR up-regulates membrane transporters *Bsep, Ntcp, Oatp2, Mrp3*, and *Mdr2*, which control the uptake of xenobiotics by the liver and the elimination of their metabolites via the kidneys or with the bile [105, 106]. Moreover, CAR is also known to induce the genes required for bilirubin clearance [74].

The specific protein structure allows CAR to interact with a wide range of substances, rendering the receptor an important player in the protective system of the organism. However, a particular interest towards CAR arose when it became evident that the molecule is involved in number of physiological and pathophysiological processes unrelated to metabolite clearance: gluconeogenesis, fatty acid metabolism, hormonal regulation, hepatocellular proliferation and hepatocarcinogenesis.

For a long time it has been known that a single administration of the potent CAR agonist TCPOBOP (1,4-bis[(3,5-dichloropyridin-2-yl)oxy]benzene) results in spontaneous hepatomegaly with a doubling of liver weight in mice [70, 107]. Meanwhile, several pathways conducive to liver growth were shown to be coordinated by CAR (Fig. 3). Firstly, activation of CAR is directly involved in the transcriptional activation of Cyclin D1 expression required for cell cycle entry [108]. Then, CAR up-regulates the expression of *Myc* and *Mdm2* [70, 75, 109]. Elevated MYC in turn enhances the expression of *Foxm1*, the essential driver of hepatocellular cell cycle progression [75]. Eventually, the increases in MDM2 and FOXM1 lead to a down-regulation of the cell cycle inhibitors P21 and P53 through several paths [110, 111]. Additionally, CAR represses P21 gene expression through direct inhibition of the P21-transcriptional promoter FOXO1 [112]. Thus, CAR stimulates various mechanisms essential for cell proliferation.

Mounting evidence supports the role of CAR in mitotic progression but also cell survival. For example, chronic induction of β-catenin results in cell aging due to a negative feedback leading to cell cycle arrest. Through the suppression of cell cycle inhibitors, CAR overrules the β-catenin-associated cell cycle arrest and counteracts cell aging [109]. Further, activation of CAR is associated with a lowered maturation of microRNA-122 (the most abundant microRNA in liver) that targets E2F1 – a transcription factor essential for cell cycle progression [113]. Moreover, TCPOBOP-induced CAR activation is associated with increased levels of YAP1, which can overrule the limits to organ size if

overactive [114]. Finally, CAR promotes survival via the transcriptional induction of *Gadd45b* [115]. Unlike its function in promoting cancer cell death, GADD45B exerts together with CAR anti-apoptotic effects in healthy liver through reduced MKK7-mediated phosphorylation of JNK7 [116].

The metabolic effects of CAR activation, namely decreased gluconeogenesis/lipogenesis and enhanced β-oxidation, were assigned to CAR's interaction with the co-activator PGC1A, eventually disrupting FOXO1-, HNF4A-, and PPARA-dependent signaling [117-119].

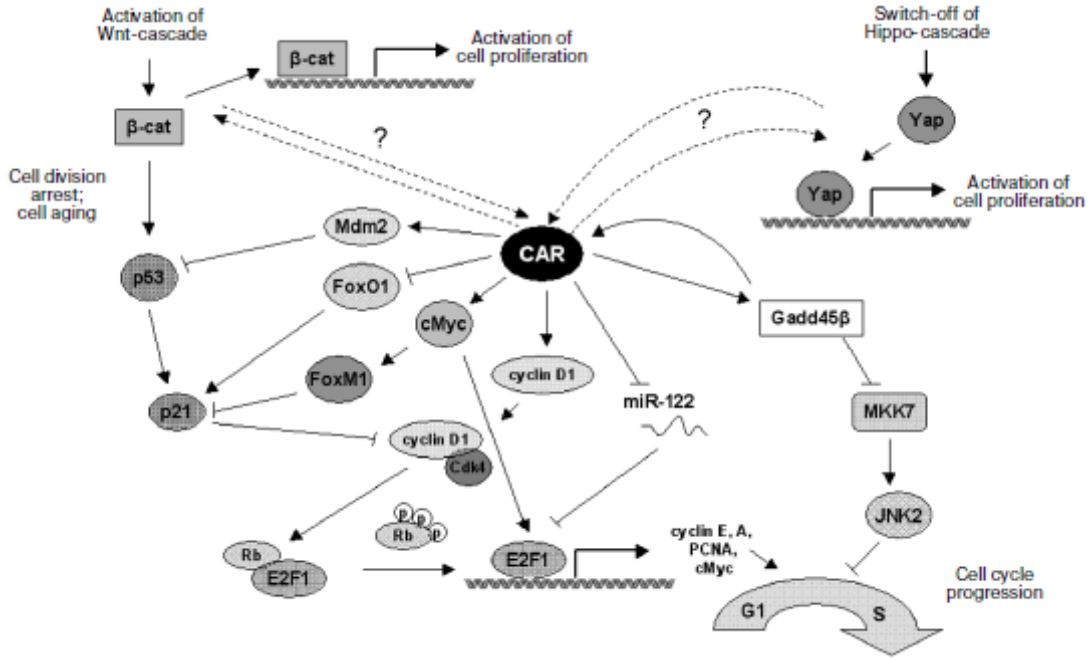

Figure 3. Summary of pro-mitotic activities of CAR. Adapted from [120].

Considering the impact of CAR on hyperplasia and energy homeostasis, it seems reasonable to hypothesize that CAR has an important function in LR. Indeed, limited data indicated delayed liver regeneration in CAR-deficient mice [26]. For my thesis, we wished to establish a promoting role for CAR in liver regeneration, with the goal to test its exogenous activation as a means to improve regeneration in the settings of resection-induced liver failure (i.e. SFSS).

To investigate the role of CAR in the SFSS development, we performed standard hepatectomies in *Car* knockout mice and assessed CAR activities after 86%-extended resection leading to liver failure in normal mice. According results suggested CAR deficiency as a contributing factor to the SFSS, owing to CAR's virtue to induce FOXM1. Using a combination of different hepatectomies, TCPOBOP-induced CAR activation and siRNA-mediated *Foxm1* knockdown, we established the importance of the CAR-*Foxm1* axis in LR and the SFSS. Further to this, we used humanized *Car* mice and *ex vivo* human liver slice cultures to estimate the clinical potential of CAR activation in liver surgery.

3.8 PTEN and PI3K/AKT pathway

PTEN (phosphatase and tensin homolog) is a well-known tumor suppressor famous for its ability to inhibit the growth-promoting AKT-mTOR pathway (Fig. 4). Because PTEN dephosphorylates PI3K-generated PtdIns(3,4,5)P3 (phosphatidylinositol (3,4,5)-trisphosphate), it inhibits phosphoinositide-dependent kinase 1 (Pdk1), which mediates the PI3K-dependent AKT activation upon growth factor stimulation [121]. Thus, PTEN controls all the downstream processes regulated by AKT-mTORC: cell proliferation, cell growth, renewal, polarity, migration and metabolism (reviewed in [122]). Accordingly, increasing evidence supports a crucial role for PTEN in the development of metabolic diseases and associated cancers. Intriguingly, hepatocyte-specific PTEN deficiency results in the rapid development of steatosis, which can progress to steatohepatitis and further to HCC [123, 124]. The loss of PTEN promotes pathological steatosis via enhanced lipogenesis and through an increased uptake of lipids [123]. Recent findings identified the transcriptional suppressor MAF1 (a FOXO1 downstream target) as an effector molecule in PTEN loss-dependent lipid metabolism and cancer signaling [125]. Importantly, *Pten* haploinsufficiency is sufficient to impair its tumor suppressing activity, indicating that even minor changes in its cellular content can have major consequences [126, 127].

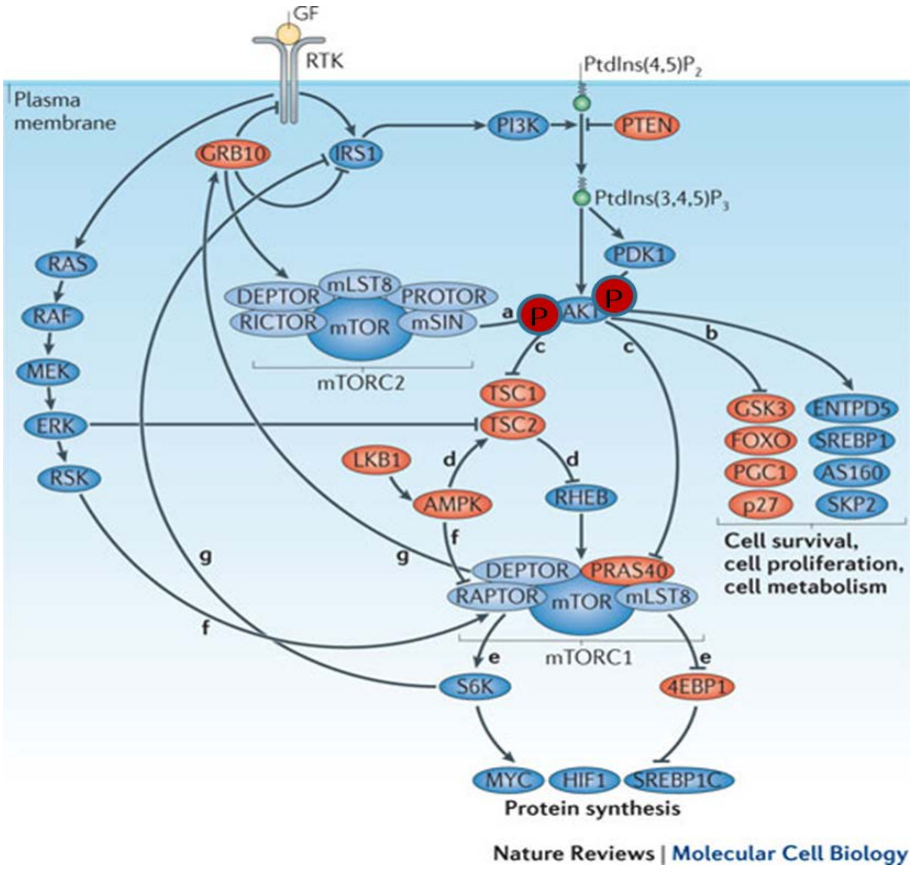

Figure 4. PTEN-PI3K/AKT pathway. Adapted from [128] with modifications.

PTEN also appears to have a role in physiological regeneration, such as shown for the regeneration of axons or pancreatic β-cells [129, 130]. Recently, PTEN was proposed to be downregulated after hepatectomy by several microRNAs, suggesting a role also in liver regeneration [131-133]. More conclusive studies have documented an important function of the AKT-mTOR axis in liver regeneration. After hepatectomy, AKT-mTOR regulates not only cellular hypertrophy, but also has an impact on glycogenesis and seemingly the formation of RAS [34, 134].

The increased activity of the AKT-mTOR axis reported in regenerating liver implies that the observed PTEN downregulation may be causally related to the activity changes after sHx. PTEN hence might be an ideal candidate to orchestrate hepatic lipid metabolism and regenerative pathways after tissue loss. We reasoned that a careful documentation of PTEN's role in liver regeneration may provide a unique opportunity to shed light onto the complex interplay between metabolic and growth-associated mechanisms that govern tissue regeneration. The major part of my PhD thesis was therefore dedicated to PTEN's function in the regenerating liver. More specifically, we first assessed PTEN levels during LR in C57/B6 mice and estimated its function via pharamcological modulation of its activities. To establish PTEN's impact on LR, we employed an inducible, hepatocyte-specific knockout model (*AlbCre*$^{tg/+}$*Pten*$^{l/fl}$ (PtenKO) and *AlbCre*$^{+/+}$*Pten*$^{fl/fl}$ (control, PtenC)). Knockout was induced shortly before hepatectomy to avoid interference of pre-existing pathological steatosis with the regenerative process. A variety of regenerative and metabolic parameters were assessed. To explore a function of PTEN in RAS, we measured substrate usage via indirect calorimetry and exposed regenerating control and knockout liver to low doses of β-oxidation inhibitors.

4. Manuscript A

Car-driven regeneration protects liver from failure following tissue loss and bears therapeutic potential

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Contribution: This study was completed during first years of the PhD. I mostly contributed to this project by performing and analyzing a number of Western blots, qPCR. A few animal experiments were performed in collaboration with Dr. Tschuor, i.e involving such techniques as extended hepatectomy and *Foxm1* knockdown. I also contributed to the manuscript revision.

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Abbreviations: Car, constitutive androstane receptor; CITCO, (6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehydeO-(3,4-dichlorobenzyl)oxime); eHx, extended hepatectomy; huCAR, humanized CAR; SFSS, Small-For-Size Syndrome; sHx, standard hepatectomy; TCP, 1,4-bis(2-(3,5-dichloropyridyloxy))benzene

Abstract

BACKGROUND & AIMS: Liver can recover following resection. If tissue loss is too excessive, however, liver failure will develop as is known from the Small-for-Size-Syndrome (SFSS). The molecular processes underlying liver failure are ill-understood. Here, we explored the role and the clinical potential of Nr1i3 (constitutive androstane receptor, Car) in liver failure following hepatectomy.

METHODS: Activators of Car, various hepatectomies, $Car^{-/-}$ mice, humanized CAR mice, human tissue and *ex vivo* liver slice cultures were used to study Car in the SFSS. Pathways downstream of Car were investigated by *in vivo* siRNA knockdown.

RESULTS: Excessive tissue loss causing liver failure is associated with deficient induction of Car. Re-activation of Car by an agonist normalizes all features associated with experimental SFSS. The beneficial effects of Car activation are relayed through Foxm1, an essential promoter of the hepatocyte cell cycle. Deficiency in the CAR-FOXM1 axis likewise is evident in human SFSS. Activation of human CAR mitigates SFSS in humanized CAR mice and improves the culture of human liver slices.

CONCLUSIONS: Impaired hepatic Car-Foxm1 signaling provides a first molecular characterization of liver that fails to recover after tissue loss. Our findings place deficient regeneration as a principal cause behind the SFSS and suggest CAR agonists may bear clinical potential against liver failure.

Key words: Small-for-Size Syndrome, TCPOBOP, CITCO, hepatomegaly

Introduction

The unique ability of liver to regenerate after tissue loss has permitted the surgical removal of large liver parts and the transplantation of partial liver grafts. The capacity of liver to regain function following tissue loss however is limited. In mice, standard hepatectomy (sHx, removal of 70% volume) leads to complete recovery within a week[1], whereas extreme resection (91% removed) induces liver failure and death within 48h[2]. Therefore, remnant volume is a key determinant for successful recovery after tissue loss.

The requirement for a sufficient liver volume is a factor significantly limiting the application of liver surgery. The transplantation of marginal liver grafts puts recipients at risk of developing liver failure, a clinical entity known as the Small-for-Size syndrome (SFSS)[3, 4]. Likewise, a congruent entity can be observed following extended hepatectomy, the most frequent intervention against highly prevalent liver tumors. In both cases, patients present with metabolic liver dysfunction (e.g. hypoalbuminemia, hyperbilirubinemia), persistent hepatostatosis, and an elevated mortality. Indeed, SFSS following liver resection or transplantation represents the most frequent cause of death due to liver surgery[3, 4].

Why small liver remnants/grafts fail to recover is incompletely understood. Following tissue loss, portal blood flow into remnants/grafts increases; an excessive elevation in portal pressure may damage the sinusoidal endothelium, eventually causing parenchymal injury, but its role in the SFSS remains controversial[5, 6]. Liver surgery often is performed with clamping of hepatic blood supply; the resulting ischemic insult (which is unavoidable in transplantation) is known to counteract liver recovery and certainly will impact marginal remnants[7]. Likewise, the accrual of injury has repeatedly been proposed to account for resection-induced liver failure in the absence of ischemia[8-10]. However, hepatectomies in mice are technically challenging and *per se* may augment liver injury[11]. To avoid confounding by surgical damage, we have introduced in mice a modified version of extended hepatectomy (eHx, 86% removed) that induces little injury as assessed by diverse parameters[1]. Despite the absence of significant injury, mice following eHx display metabolic liver dysfunction, hepatosteatosis and an elevated mortality akin to human SFSS[1]. Therefore, injury is not required for liver failure to develop after extended tissue loss in mice.

Our experimental SFSS model further was associated with delayed regeneration due to arrest at the S and particularly M phase of the hepatocyte cell cycle. When repeating eHx in mice lacking the generic cell cycle inhibitor p21, liver regeneration was restored and most metabolic SFSS features were ameliorated, as was survival[1]. These improvements suggest an impaired regenerative capacity may suffice to cause SFSS.

Regenerative deficits indeed are a consistent finding in models of resection- or transplantation-induced SFSS[8-10, 12]. The road to impaired hepatocyte proliferation however remains ill-understood, and no clear-cut molecular defects are known for human SFSS. The notion that impaired regeneration and metabolic dysfunction go hand in hand with a marginal liver volume may hint to a pathway that coordinates hepatocyte proliferation with the liver's metabolic tasks. Nr1i3 (constitutive androstane receptor, Car) is a nuclear receptor that regulates P450 cytochromes and has diverse metabolic functions[13], including the clearance of xeno/endobiotics such as toxic bilirubin[14]. Notably, Car activation through phenobarbital-like agents induces spontaneous hepatomegaly[15]. Likewise, Car appears to be required for liver regeneration after hepatectomy[16].

To this end, we investigated (i) whether disturbed Car-dependent signaling is associated with the development of liver failure after tissue loss in mice, (ii) whether putative deficits are relevant for human SFSS, and (iii) whether Car modulation may be exploited for the clinical management of SFSS.

Materials & Methods

Animals

Animals aged 8-10 weeks were kept on a 12-hour day/night cycle with free access to food and water. Male wild type mice (C57BL6, Harlan) were used unless otherwise stated. CAR knockout animals (9103-M, C57BL/6-*Nr1i3tm1.1Arte*) and corresponding wild type controls were obtained from Taconic Laboratories, as were humanized CAR mice (9101-M, C57BL/6-*Nr1i3tm1(NR1I3)Arte*). Due to local requirements, breeding was started with offspring from in-house C57BL6 following embryonic transfer.

Animal Surgery

Standard hepatectomies (sHx, 70%, fully regenerating, 100% survival) and extended hepatectomies (eHx, 86%, regenerative delay, >75% survival, SFSS model) were performed as reported[1]. The same surgical technique was applied for extreme hepatectomy (91%, 0% survival within 48h), except that all segmental portal vessels of the right, left, and middle lobes were ligated. Sham operation consisted of cholecystectomy. SFSS orthotopic partial liver transplantations (using 30% (v/v) grafts) were performed according to Tian *et al.*[17]. The gain in liver weight, a physical measure of liver regeneration, was expressed through the ratio of liver weight to body weight (LW/BW).

Activation of mouse Car and human CAR

BL6 and *Car* knockout mice were treated with the murine Car agonist TCP (1,4-bis(2-(3,5-dichloropyridyloxy))benzene, Sigma Aldrich) directly prior to surgery or as indicated (see Supplementary Figure 1A for TCP effects in the absence of surgery). TCP was dissolved in DMSO (5mg/ml), mixed with prewarmed PBS (final vol. 100µl) and given by oral gavage (1-3mg/kg). The human CAR agonist CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichloro-benzyl)oxime, Sigma Aldrich, C6240, dissolved in DMSO at 5mg/ml*)* was i.p. injected with prewarmed PBS (final vol. 100µl) into humanized *CAR* (huCAR) mice at 50mg/kg directly before hepatectomy and then daily until harvest. For *ex vivo* liver slice cultures, 250nM TCP and 1 or 100µM CITCO were added to mouse and human media, respectively.

Foxm1 knockdown

siRNAs targeting *Foxm1* and the controls *Aha1*and *Luciferase* were designed by Axolabs Gmbh (Kulmbach, D) and packed into company-owned formulations designed to preferentially target murine hepatocytes. Formulations were injected into the tail vein 48 hours before hepatectomy. The lack of significant toxicity was ascertained through the assessment of liver injury markers.

Immunochemistry and tissue microarray

These techniques were performed according to standard protocols and are described in the Supplements, including a description of human biopsy material.

Western Blotting

The procedure was performed as reported[1]. Antibodies are described in the Supplements.

Quantitative Real-Time Polymerase Chain Reaction

Sequence amplification and data analysis were performed on the ABI Prism 7000 Sequence Detector System (PE Applied Biosystems) as detailed in the Supplements. If not otherwise stated, expression values were normalized to time-matched samples from sham-operated mice.

Ex vivo culturing of liver slices

Ex vivo cultures of liver slices were prepared as described by de Graaf *et al.*[18] with slight modifications. Liver biopsies were obtained from three mice (C57BL6) and one human subject (diagnosed with colorectal liver metastasis, tissue from an unaffected lobe). Biopsies were embedded in 10ml liquid (2%wt/vol) ultralow-melting-point agarose dissolved in Krebs-Henseleit buffer (KHB, 5mM NaCl, 118mM KCl, 1.1mM $MgSO_4 \cdot 7H_2O$, 1.2mM KH_2PO_4, 25mM $NaHCO_3$, 2.5mM $CaCl_2 \cdot 2H_2O$, 25mM D-Glucose, 9mM HEPES in ultrapure water), cut with a vibratome into 200µm thick slices and kept in KHB. Five to six slices were then plated on 0.4uM inserts (30mm diameter, Millicell) and the residual buffer was removed before placing the inserts into a cell culture plate containing culturing medium (Williams E medium (+L-glutamine)

supplemented with antibiotics and 14mM D-glucose (Sigma), 30nM insulin (Gibco Life Technologies), 100nM glucagon (Sigma), 1nM corticosterone (Sigma), 1nM Egf (Sigma) plus 5% FCS. The tissue cultures were kept in a standard cell incubator (37°C, 5% CO2 and 95% humidity) and the medium was changed daily. Integrity of explants was assessed by HE staining (i.e. the presence of a nucleus and a normal cell structure on histology). Experiments were limited to 24/48h cultures due to inconsistent tissue integrity at later times. Experiments with liver biopsies from three mice and one human subject were run in triplicates. Ethical approval for the use of human biopsy tissue was granted by the local ethics committee from Zürich (KEK-ZH-Nr. 2012-01 08).

Statistical analysis

Data are presented as mean ±SD. Differences between the groups were assessed by a two-tailed *t-test* assuming unequal variance. In general, at least 5 mice/group were analyzed. For survival after sHx/eHx in wt/ $Car^{-/-}$ mice and *Foxm1* knockdown, 10 animals/group were included. For the molecular analyzes following siRNA knockdown, at least three mice/group were used. Differences were considered significant at $P<0.05$ and indicated in figures by an asterisk (*). Statistical analyzes were performed using Prism 6.0 (GraphPad).

Study approval

All animal experiments were in accordance with Swiss Federal Animal Regulations and approved by the Veterinary Office of Zurich. Ethical approval for the human sections was granted by the regional ethics committee (KEK-ZH -Nr. 2012-01 08). Written consent to study tissue for research purposes was received from the patient prior to inclusion in the study.

Results

Car activation after eHx is defective. To assess Car activity following resection, we measured its mRNA levels, protein levels/localization and downstream targets following resection. Compared to sHx, Car mRNA induction and nuclear localization were impaired after eHx (Figure 1*A*, Supplementary Figure 1*B*). Likewise, the prototypical Car target *Cyp2b10*[13] and the proliferation-related downstream molecule Foxm1[19] were hardly induced (Figure 1*A*, Supplementary Figure 1*C*), indicating defective Car activation following eHx.

Car deficiency causes an SFSS phenotype after sHx. Car is thought to be needed for efficient liver regeneration[16]. To detail its function in regeneration, we analyzed $Car^{-/-}$ mice following resection and compared to wild type (wt) mice post sHx/eHx. Although usually at 100%, survival after sHx in $Car^{-/-}$ mice was reduced to levels comparable to eHx in wt mice (Figure 1*B*). Liver weight gain, pH3 staining, mitoses, and *Cyp2b10* expression were decreased compared to wt sHx. Similarly, *Foxm1* and its cyclin targets *Ccna2/b2* were reduced, whereas *Cdkn1a* (p21, repressed by Foxm1)[20] was upregulated; the SFSS-associated hepatosteatosis (see also Supplementary Figure 1*D*) and metabolic liver dysfunction (hypoalbuminemia, hyperbilirubinemia) were present (Figure 1*B*). Therefore, sHx in $Car^{-/-}$ mice induces a phenotype akin to eHx in wt mice. Together with impaired Car activity post eHx, these results identify Car deficiency as a cause of experimental SFSS.

Car re-activation rescues from SFSS. Car ligands such as phenobarbital-like compounds induce Car activity[15]; TCP (1,4-bis(2-(3,5-dichloropyridyloxy))benzene) is the most potent agent of this class, with one TCP gavage nearly doubling mouse liver weight within nine days[21]. We found TCP-induced hepatomegaly was accompanied by Car nuclear accumulation/downstream gene induction (Supplementary Figure 1*A*). To determine whether TCP is able to re-activate Car after eHx, TCP was given to mice concomitant with eHx. One gavage was sufficient to restore Car nuclear translocation (see also Supplementary Figure 1*B*) and downstream gene induction. Moreover, TCP suppressed p21, re-elevated pH3 and mitotic counts, accelerated liver weight gain, and normalized metabolic SFSS features after eHx (Figure 1*C*). TCP lost these effects in $Car^{-/-}$ mice, confirming dependency on Car

(Supplementary Figure 2). Next, we assessed the impact of TCP on survival. Given that most mice (>75%) survive eHx-induced SFSS, TCP was tested in an alternative SFSS model featuring 0% survival (91% hepatectomy). One-week-survival was assessed, as this is the critical period after hepatectomy. TCP rescued 40% of mice after 91% hepatectomy. When TCP was tested in another lethal SFSS model (transplantation of 30% (v/v) SFSS grafts)[17], it again raised survival to 40% (Figure 1*D*). These findings demonstrate that Car reactivation through TCP leads to a functional recovery of marginal liver remnants and grafts.

Foxm1 mediates Car effects in experimental SFSS. TCP-induced hepatomegaly (Supplementary Figure 1) is paralleled by *Foxm1* induction[19], however the dependency of Car effects on Foxm1 remains unexplored. On the other hand, sHx in *Foxm1*$^{HEP-/-}$ mice induces delayed progression through the S- and M-cell cycle phases, akin to eHx in wt mice[1, 20]. To determine whether Foxm1 may be associated with SFSS, we mimicked its deficiency by α*Foxm1*-siRNA-mediated knockdown before sHx. Knockdown was observed during the S and M phase peaks (32h and 48h, respectively, Figure 2*A*, Supplementary Figure 1*E*) when Foxm1 is maximally induced after sHx (Figure 1*A*, Supplementary Figure 1*C*). *Foxm1* knockdown reduced proliferative parameters, diminished liver weight gain, and caused hypoalbuminemia, hyperbilirubinemia and hepatosteatosis (see also Supplementary Figure 1*D*). Together with the compromised survival following knockdown and sHx (Figure 2*A*), these findings indicate a crucial contribution of Foxm1 deficiency to the development of SFSS. When knocking down *Foxm1* before eHx, TCP lost its effects, with liver remnants remaining small and steatotic (Figure 2*B*, Supplementary Figure 1*D*). We conclude that Car activation via TCP requires signaling through Foxm1 to prevent the development of experimental SFSS.

Human SFSS displays pathobiological changes akin to mouse SFSS. Human SFSS has not yet been investigated at a molecular level. We analyzed liver tissue from SFSS patients and those without complications after resection. Regenerating human livers, but not SFSS livers, were positive for nuclear CAR and FOXM1 (Supplementary Figure 3). Both regenerating and SFSS livers expressed KI67, indicating hepatocytes have entered the cell cycle[1]. In contrast, p21 was induced whilst pH3 was hardly detectable in SFSS livers, consistent with deficient cell cycle progression. Human and mouse SFSS thus seem to share basic pathophysiological mechanisms, implying the activation of human CAR might prevent SFSS in the clinic.

CAR activation for human SFSS. Because TCP has little activity towards human CAR[22], we examined the human CAR agonist CITCO (6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde*O*-(3,4-dichlorobenzyl)oxime)[23] for its ability to prevent experimental SFSS in transgenic mice bearing a human CAR (huCAR mice, where mouse Car has been replaced with human CAR)[14]. In response to CITCO alone, huCAR mice developed spontaneous hepatomegaly (Figure 3*A*), albeit less pronounced than wt mice on TCP. In fact, huCAR mice following sHx displayed impaired regeneration accompanied by metabolic SFSS features and increased mortality, indicating that huCAR mice do not retain the full regenerative capacity of wt mice (Figure 3*B*). Nonetheless, CITCO improved liver weight gain, pH3 counts, steatosis and hyperbilirubinemia in huCAR mice after eHx (Figure 3*C*). To provide further evidence for the clinical benefit of CAR activation, we treated *ex vivo* cultures of liver slices from a patient biopsy. Similar to mouse liver slices on TCP (Figure 4*A*), CITCO improved histology (see also Supplementary Figure 4) and increased pH3 counts in human slices (Figure 4*B*). Importantly, CITCO at high doses also improved viability (AST, Hmgb1, Casp3), but was less potent than TCP. Therefore, CITCO mitigates liver failure in huCAR mice and exerts proregenerative and protective effects on human liver slices, suggesting CAR activation may be effective against human SFSS.

Discussion

In this study, we demonstrate a vital role for the nuclear receptor Car in the development of liver failure following tissue loss. Unlike in normally regenerating liver, Car is not activated following extended resection of the liver. Standard hepatectomy in mice lacking Car provokes a phenotype akin to that seen in our SFSS model after eHx. A key consequence of Car deficiency is the failed induction of the cell cycle promoter Foxm1, knockdown of which is sufficient to induce most SFSS features following sHx. Re-activation of Car through its species-specific ligand TCP normalizes all of these features, illustrating the importance of Car activity for the prevention of experimental SFSS.

The association between Foxm1-dependent cell cycle deficits and the SFSS phenotype suggests delayed liver regeneration is the underlying cause of resection-induced liver failure. Foxm1 is considered a proliferation-specific transcription factor also in hepatocytes[24][20]. Foxm1 knockdown not only induced an SFSS-like phenotype, but also blunted the rescuing effects of TCP. As a limitation, only one siRNA against Foxm1 was used, and off-target effects hence cannot be excluded. However, Foxm1 knockdown before sHx causes cell cycle defects similar to sHx in hepatocyte-specific Foxm1 knockouts[20], sHx in *Car*$^{-/-}$ mice, or eHx in wt mice[1]. We therefore propose that delayed progression through the S and particularly M cell cycle phase is a basic cause of liver failure.

Besides regulating Foxm1, the functions of Car in hepatic metabolism will add to the full prevention of an SFSS phenotype. Simply due to volume loss, marginal remnants may be overwhelmed by the metabolic needs posed on liver. Acting as a xeno/endobiotic sensor[13], Car may react via Foxm1 to augment liver mass, but also by inducing a panel of metabolizing enzymes, including those needed for the clearance of elevated bilirubin levels[14]. The unusual ability of Car to modulate organ size restraints while controlling hepatocyte proliferation and function[25] implies its main task is the coordination between liver volume, its metabolic capacity, and the current metabolic demands. These qualities, and the possibility to induce its activity by exogenous ligands, render Car an attractive candidate for the mitigation of SFSS in the clinic.

For Car to be a clinically viable target, (i) human SFSS should display functional deficits in the Car axis, (ii) activation of human CAR should lead to similar outcomes as with mouse Car, and (iii) clinical situations should be amenable to CAR-based strategies. The alterations we observed in human SFSS liver were consistent with a deficient CAR-FOXM1 axis along with defective cell cycle progression. As for the activation of human CAR, the human agonist CITCO was tested in two different systems, huCAR mice and *ex vivo* human liver slices. Although huCAR mice have been reported to efficiently induce Car-dependent metabolic enzymes[14], the CITCO responses we observed (i.e. the development of mild SFSS following sHx, volatile CAR downstream gene expression patterns) suggest a subpar communication between human CAR and its downstream mouse partners, perhaps rooting in the protein structure considerably differing between man and mouse (huCAR/mCar size ratio=1.66, with 70% identity only in the common sequence (http://www.uniprot.org). These observations however also emphasize the need of full Car activity for the prevention of liver failure. Despite the above inadequacies, CITCO did mitigate SFSS in huCAR mice and efficiently improved liver weight gain, likely owing to the induction of Foxm1. Similar to TCP, CITCO also was able to improve the integrity of human liver slice cultures and promote their proliferation. Again, CITCO was less effective than TCP; unlike TCP, repeated injection of CITCO is required to induce a response *in vivo*[26], indicating a relatively low efficacy of the ligand. Novel CAR ligands with improved potency/stability will likely be needed to achieve full activation of human CAR. The effects of CITCO on huCAR mice and human liver slices however provide a proof-of-concept for the potential clinical utility of CAR activation.

Apart from remnant/graft volume and the presence of pre-existing liver disease[3, 4], no clinical predictors of SFSS currently exist,

placing treatment over prevention. To estimate the therapeutic potential of Car activation, we applied TCP to our lethal SFSS model in a delayed mode (i.e. *after* surgery). Indeed, TCP maintained its beneficial effects and again rescued 40% of mice (Supplementary Figure 5), implying a therapeutic window exists for the rescue from SFSS. Another obstacle to clinical translation is the malignant potential associated with CAR activation. The prime indication for extended hepatectomies is liver malignancy, and Car ligands are non-genotoxic promoters of rodent liver tumors[27]. CAR activation hence might potentially increase the risk of recurrence, however both TCP and Car activation are associated with malignancy only in chronic settings, suggesting a single application bears little risk [27, 28]. No according data is available for human CAR, however phenobarbital treatment seems not to increase liver cancer incidence in patients[29]. To minimize risks, putative trails might focus on hepatectomies for liver cancer displaying CAR downregulation (Supplementary Figure 6). Given that TCP was likewise efficient in a model of SFSS transplantation, live liver donors may safely benefit from CAR activation, i.e. for the treatment of SFSS developing in donors following partial graft retrieval, or for a pre-operative enlargement of donor liver size to enable the riskless removal of sufficient volume for transplantation. Finally, unlike human liver, mouse liver is composed of distinct lobes that can be resected for hepatectomy. The more compacted architecture of human liver however usually requires parenchymal transection for hepatectomy, causing liver injury. Although injury is not necessary for SFSS to develop, it will increase the SFSS risk. Therefore, effective clinical strategies could be based on a combined approach to promote the regenerative capacity (i.e. CAR) and to prevent injury. CAR activation itself has some protective effects, however adding compounds specifically targeting injury may be more effective[10].

Taken together, our study identifies Car deficiency as a key mechanism underlying the development of liver failure following extended tissue loss, and provides the means to correct these deficits. The function of Car in hepatic regeneration points to the translational potential of CAR activation, creating demand for novel, powerful agonists of human CAR. Moreover, the dependency of the beneficial Car effects on cell cycle-associated molecules imply that liver failure, including many of its metabolic features, arise from an insufficient capacity of marginal remnants to regenerate, illustrating the intimate nexus between proliferative pathways and the metabolic function of liver.

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Author's contributions

CT, BH and RG designed the experiments. CT, EK, LP, DAR, and ML performed molecular analyses. KG performed immunohistochemistry. YT performed mouse liver transplantations. UH provided expertise for the *ex vivo* experiments. AW evaluated human liver samples and contributed to tissue array studies. AC provided intellectual input and critically revised the manuscript. BH designed the study. BH, CT, RG and PAC wrote the manuscript. BH, RG and PAC supervised the study. The authors have no conflicts of interest.

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Figure legends

Fig.1. Car deficiency underlies liver failure after eHx. (A) Car gene/protein expression and downstream gene expression after eHx. Despite similar protein levels, Car nuclear accumulation is impaired after eHx. (B) sHx in $Car^{-/-}$ mice induces an SFSS phenotype, as evinced through the assessment of relevant SFSS parameters (at 48h post sHx/eHx for wt mice and post sHx for Car-/- mice). Note that LW/BW (liver-to-body-weight ratio) cannot be compared between sHx and eHx due to a different starting value. For survival, 10 mice/group were used. (C) TCP reactivates Car and normalizes SFSS features (shown for 48h post Hx) after eHx. TCP-induced Foxm1 re-elevation at 32/48h post eHx is illustrated in a magnified square. (D) TCP improves survival in lethal SFSS models. N=5/group unless otherwise stated; *P<.05.

Fig. 2. The beneficial effects of TCP in SFSS rely on signaling through Foxm1. (A) Foxm1 knockdown before sHx provokes SFSS-like features. Control siRNAs against *Luc* and *Aha1* (shown for 48h only) were used. Note the comparison to plain sHx/eHx for LW/BW, albumin and bilirubin. Survival following sHx was reduced by *Foxm1*-siRNA, reflecting compromised liver function. (B) Foxm1 knockdown prior to eHx abrogates the beneficial effects of TCP. In the siRNA-treated samples, (-) indicates a control without siRNA. Note the small size and the pale complexion (typifying steatosis) of liver remnants after eHx, or eHx plus TCP following *Foxm1*-siRNA-pretreatment. N≥3/group for molecular analyses, n=10/group for survival, otherwise n=5/group.

Fig. 3. CITCO induces spontaneous hepatomegaly and mitigates most SFSS features in huCAR mice. (A) Spontaneous hepatomegaly in huCAR mice through CAR activation via CITCO as evinced through the assessment of SFSS parameters. Note upregulation of *Cdkn1a*, and the marginal elevation in *Ccna2/b2*. (B) sHx in huCAR mice induces a mild SFSS phenotype. Note the reduced LW/BW and survival, the high gene expression, and elevated steatosis/bilirubin in huCAR mice. (C) CITCO mitigates liver failure in huCAR mice after eHx. Note the improvements in LW/BW, *Foxm1*, *Cdkn1a*, *Ccna2/b2*, pH3 and bilirubin, and the lack of significant effects on steatosis and albumin through CITCO. N=5/group.

Fig. 4. CAR activation promotes the proliferative state and the integrity of *ex vivo* liver slice cultures. (A) TCP effects in mouse liver slices cultured for 24h or 48h. TCP added to media improves liver histology (HE: appearance of nuclei and regular cell structure, see also Supplementary Figure 4), promotes nuclear pH3 positivity, accompanied by reduced supernatant levels of injury markers AST/Hmgb1. Staurosporin served as positive injury control. (B) CITCO effects in human liver slices. At 1µM, CITCO induces modest improvements in histology and proliferative markers. At 100µM, these effects are stronger, along with reduced apoptotic counts (Casp3) and Hmgb1 supernatant levels. For ex vivo culture, three slices from each three mice, and five slices from one human liver biopsy were analyzed.

Figures

Figure 1

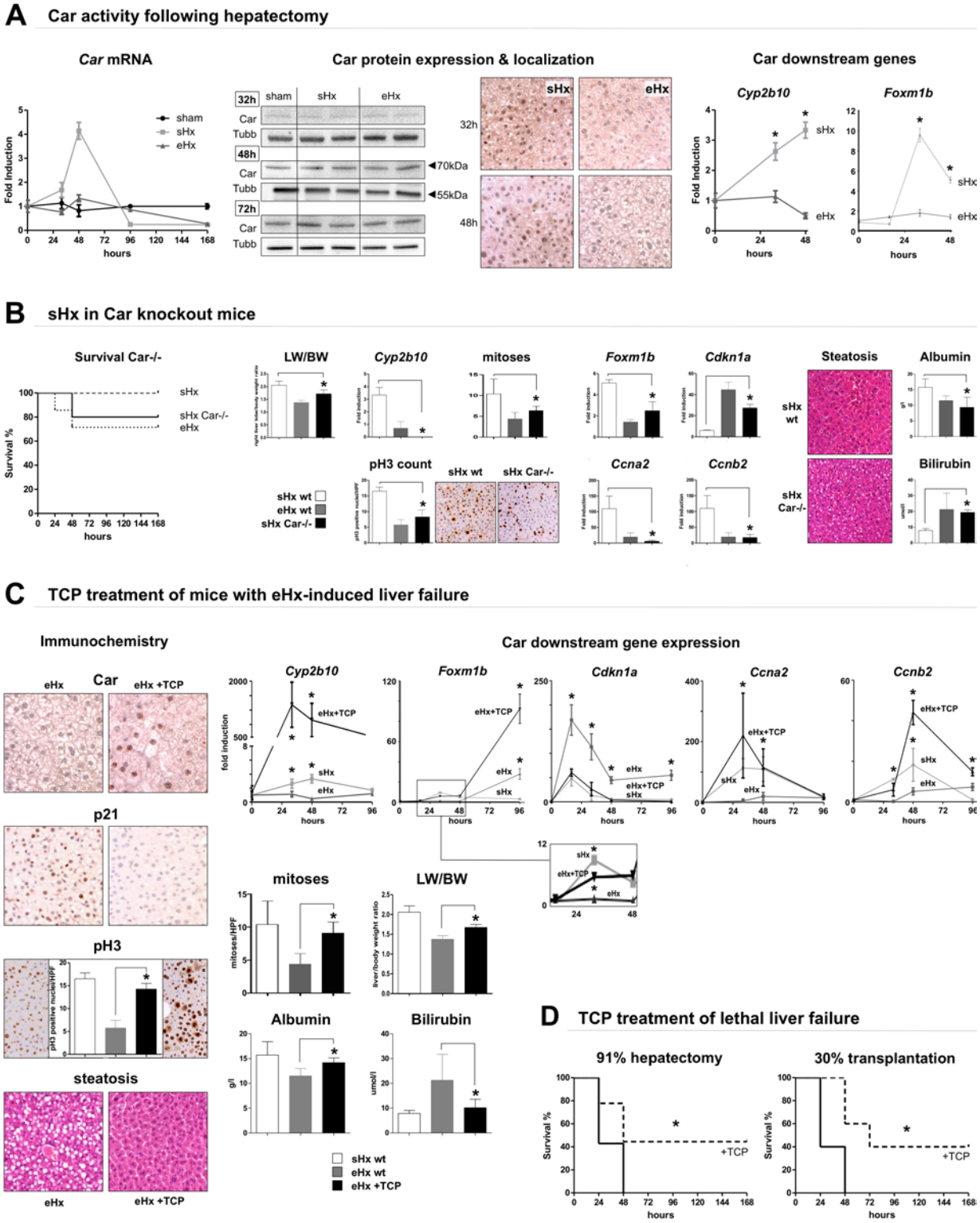
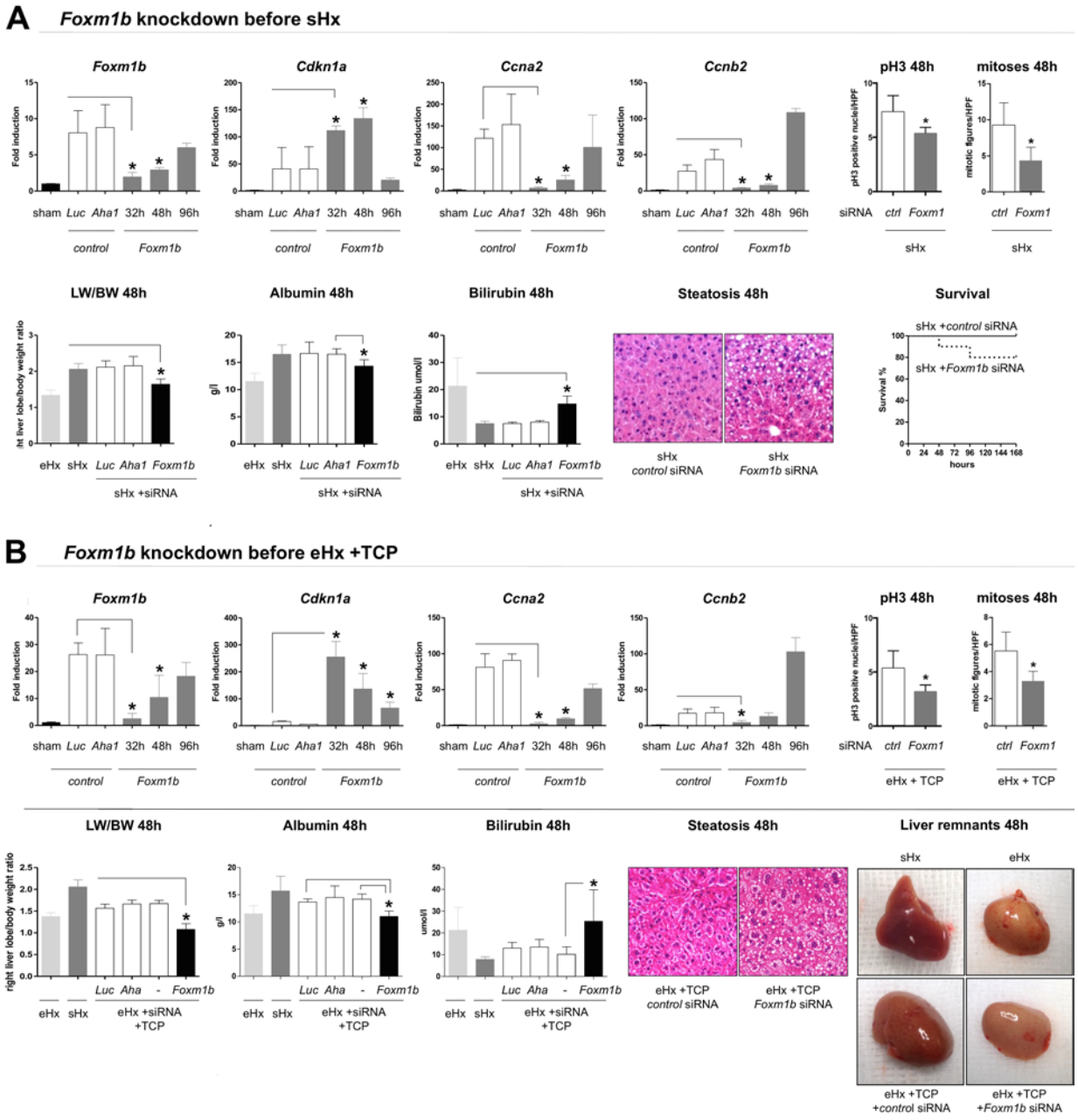


Figure 2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Foxm1b knockdown before sHx
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mitotic figures/HPF
sham Luc Aha1 32h 48h 96h
control Foxm1b
siRNA ctrl Foxm1
ctrl Foxm1
sHx
LW/BW 48h
Albumin 48h
Bilirubin 48h
Steatosis 48h
Survival
lht liver lobe/body weight ratio
g/l
Bilirubin umol/l
Survival %
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sHx control siRNA
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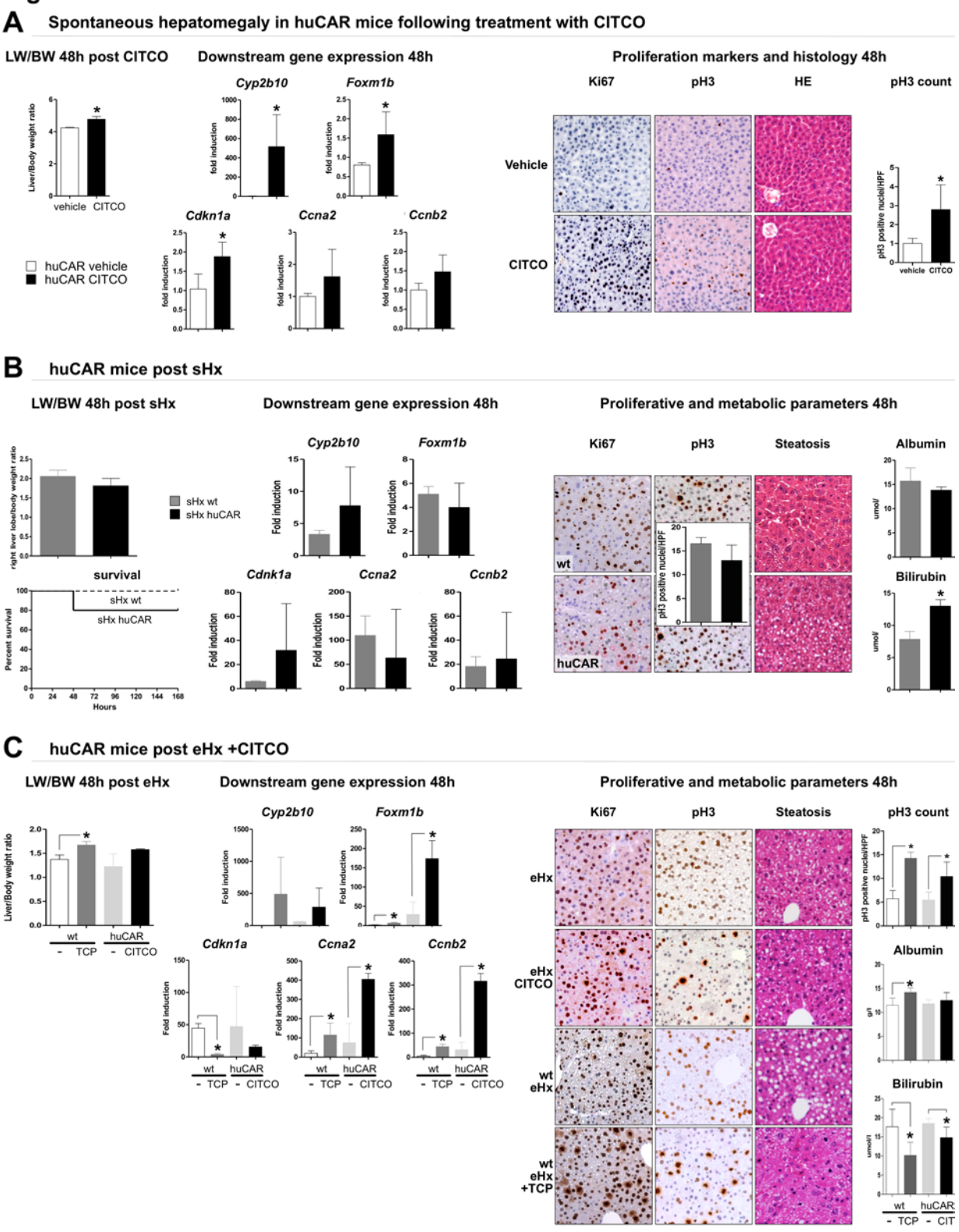
Figure 3


A
Spontaneous hepatomegaly in huCAR mice following treatment with CITCO
LW/BW 48h post CITCO
Liver/Body weight ratio
vehicle
CITCO
huCAR vehicle
huCAR CITCO
Downstream gene expression 48h
Cyp2b10
Foxm1b
Cdkn1a
Ccna2
Ccnb2
fold induction
Proliferation markers and histology 48h
Ki67
pH3
HE
pH3 count
Vehicle
CITCO
pH3 positive nuclei/HPF
B
huCAR mice post sHx
LW/BW 48h post sHx
right liver lobe/body weight ratio
sHx wt
sHx huCAR
survival
Percent survival
Hours
Downstream gene expression 48h
Fold induction
Proliferative and metabolic parameters 48h
Steatosis
Albumin
Bilirubin
wt
huCAR
C
huCAR mice post eHx +CITCO
LW/BW 48h post eHx
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eHx
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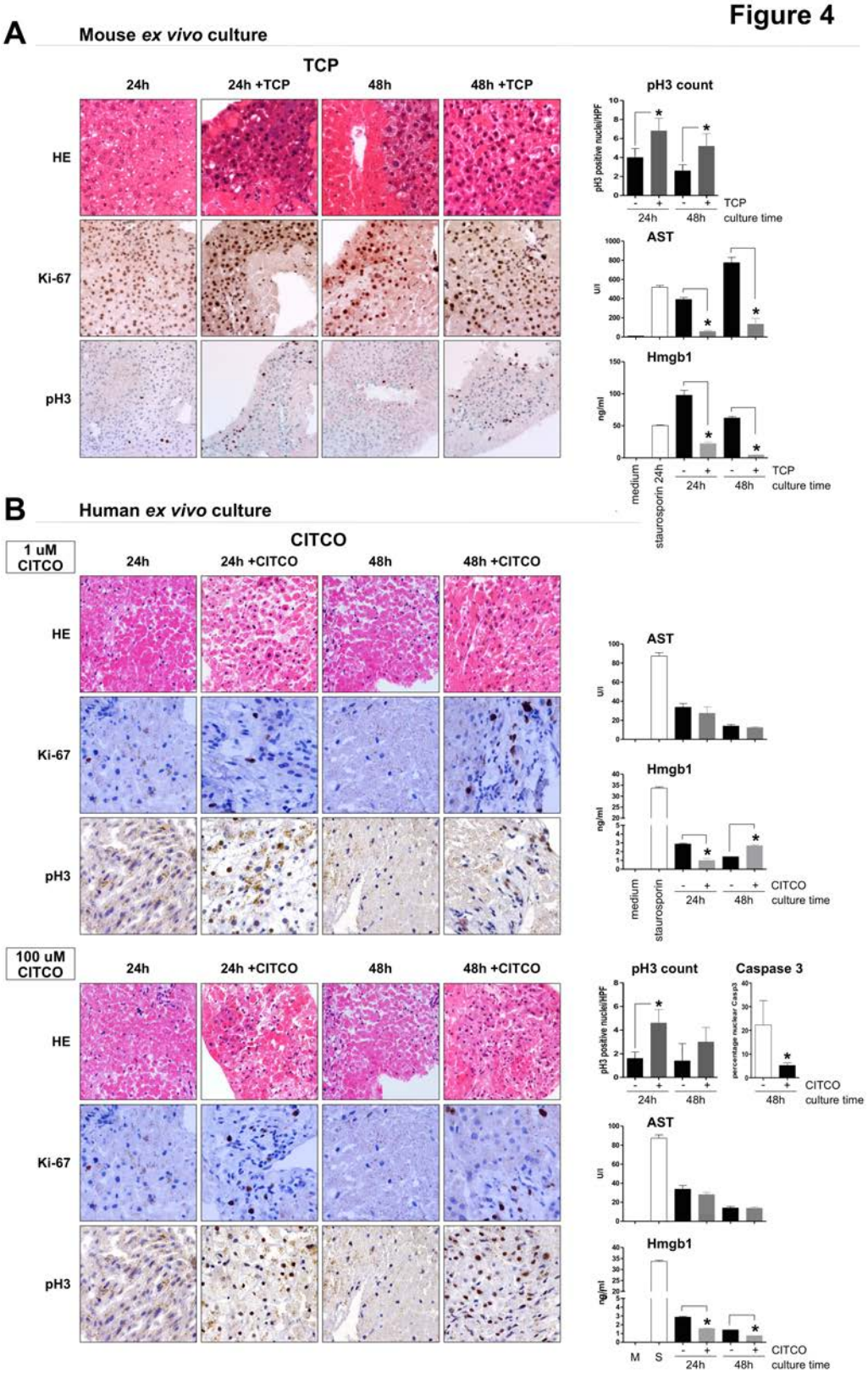
wt eHx
wt eHx +TCP

Figure 4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24h
24h +TCP
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48h +TCP
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Ki-67
pH3
pH3 count
pH3 positive nuclei/HPF
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culture time
AST
UI
Hmgb1
ng/ml
medium
staurosporin 24h
B
Human ex vivo culture
CITCO
1 uM CITCO
100 uM CITCO
Caspase 3
percentage nuclear Casp3
M
S

Supplement

- *Supplementary Materials & Methods*

- *Supplementary Figures 1-6*

Materials & Methods

Immunochemistry and tissue arrays

Immune stainings were performed on 3 µm formalin-fixed, paraffin-embedded liver sections. Antigenes were retrieved by boiling in citrate buffer. The following primary antibodies were used: Ki67 (Abcam 16667), pH3 (Abcam ab92628), p21 (BD Pharmingen 556431), Plin2 (Abcam ab181463), Foxm1 (Santa Cruz sc-32855) and Car (LifeSpan BioSciences LS-C30862). Secondary detection was done using the Ventana Discovery automated staining system and the iView DAB kit (Ventana Medical Systems). Immunostainings on human tissue (CAR (Santa Cruz sc-13065), P21 (BD Pharmingen 556431), pH3 (Abcam ab92628), KI67 (Lifescreen Ltd. CMC27531021), FOXM1 (Santa Cruz sc-32855) and a part of the mouse tissue stainings were performed by Sophistolab AG (Muttenz, CH). Ki67 and pH3 positivity was assessed by manual counting in 10 random visual fields. Biopsy tissue was obtained from potential living liver donors (*n=5, normal liver*), from hepatectomy patients (including resectate samples) without postoperative complications (*n=7, normally regenerating liver, retrieved at day 7 (3), day 8 (1), day 9 (2) and day 11 (1) post surgery*), and from hepatectomy patients that had developed SFSS (*n=7, SFSS, retrieved at day 5 (1), day 7 (1), day 9 (2), day 10 (1), day 12 (1), day 14 (1) post surgery*). The clinical diagnosis of SFSS was based on the '50-50 criteria' (Balzan *et al.* The "50-50 criteria" on postoperative day 5: an accurate predictor of liver failure and death after hepatectomy. Ann Surg 2005;242:824-828). Tissue microarray staining for CAR (LS-C30862) was performed in the Institute of Surgical Pathology at the University Hospital Zürich. Histological analyses were performed in a blinded fashion.

Western Blotting

The following primary antibodies were used: Car (Santa Cruz, sc-13065) and β-tubulin (Cell Signalling, 2128).

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from 20 mg of liver tissue using TRIzol reagent (Invitrogen) and transcribed into cDNA using the ThermoScript reverse-transcription PCR System (Invitrogen). TaqMan gene expression assays for *Car (*Mm01283978_m1), *Cyp2b10* (Mm00456591_m1), *Ccna2* (Mm00438064_m1), *Ccnb2* Mm01171453_m1, *Cdkna1* (Mm00432448_m1), *Foxm1* (Mm00514924_m1) and *18S rRNA* internal control (TaqMan ribosomal RNA control reagents) were from PE Applied Biosystems; PE Applied Biosystems). The results shown represent fold induction of mRNA expression ± SD.

AST, albumin, bilirubin and Hmgb1 levels

Serum samples were obtained from the inferior vena cava before organ harvesting. AST, albumin and bilirubin levels were measured using a serum multiple biochemical analyzer (Dri-Chem 4000i, Fujifilm). Hmgb1-ELISA was from Shino Test.

Supplementary Figure legends

Supplementary Figure 1. Supplementary information for figures 1 and 2. (*A*) Car activation via TCP induces spontaneous hepatomegaly. A single TCP administration leads to liver growth (liver-to-body weight ratio) and nuclear accumulation of Car accompanied by increases in pH3 counts. The elevation in Car activity through TCP is reflected in the induction of its downstream genes *Cyp2b10* and *Foxm1* (expression normalized to vehicle-treated, time-matched samples). N=3/group; **P*<.05. (*B*) Quantification of Car deficiency after eHx and its correction through TCP. N=5/group. Control immunohistochemistry shows Car staining in kidney (where expression is very low) and a negative liver control without primary antibody. (*C*) Foxm1 deficiency after eHx. Immunohistochemistry confirms deficient induction of nuclear Foxm1 protein after eHx. Three mice/group were analyzed. (*D*) Plin2 staining for confirmation of steatotic changes. Staining for Plin2, a membrane protein required for the formation of lipid vesicles, marks fat droplets in liver and corroborates the steatotic alterations seen on histology (Figs. 1&2) following sHx, eHx, and eHx plus TCP. Three mice/group were analyzed. (*E*) Efficacy and tissue-specificity of Foxm1 knockdown at the protein level. Knockdown of *Foxm1*, but not *Aha1* or *Luc*, causes nuclear Foxm1 depletion in regenerating liver. Note the absence of nuclear Foxm1 expression in non-parenchymal liver cells (see also *C*). Although Foxm1 was also expressed at moderate levels in the colon and lung of mice after Hx, no expression differences were noted for these tissues following Aha1, Luc, or Foxm1 knockdown, suggesting that the siRNA formulation preferentially targets hepatocytes. Three mice/group were analyzed.

Supplementary Figure 2. TCP remains without effect in $Car^{-/-}$ mice. (*A*) TCP does not induce spontaneous hepatomegaly in $Car^{-/-}$ mice. Liver weight (LW/BW) and Car nuclear expression are shown for day 3 following TCP (3mg/ml) gavage. Car downstream gene expression likewise is not induced at day 3 after TCP injection in both naive and sham-operated liver of $Car^{-/-}$ mice. (*B*) TCP does not improve SFSS-related parameters in $Car^{-/-}$ mice. LW/BW, Car immunohistochemistry, downstream gene expression, pH3 staining, cell cycle-associated gene expression, and metabolic parameters are shown for 48h post eHx. Hepatectomized wt mice with or without TCP treatment are included for comparison. Note the lack of differences between TCP-treated and untreated $Car^{-/-}$ mice. N=5/group; **P*<.05.

Supplementary Figure 3. Pathobiology of human SFSS. (*A*) CAR activation is deficient in human SFSS. CAR immunohistochemistry for normal (non-regenerating), regenerating (after hepatectomy) and SFSS (after extended hepatectomy) is shown. CAR nuclear positivity counts were 5±3/HPF in normal, 318±47/HPF in regenerating, and 54±33 in SFSS liver. Note the presence of lipid droplets in SFSS liver. (*B*) CAR-dependent processes are defective in human SFSS. FOXM1, P21, pH3 and KI67 immunostainings are shown for regenerating and SFSS liver. Note that visualization of P21 was done using an alkaline-phosphatase-based red chromogenic substrate. Five and seven patients with regenerating and SFSS liver, respectively, were analyzed (see Supplementary Methods for patient details). Stainings for the same set of molecules in mouse liver after sHx (normally regenerating, n=5) or eHx (experimental SFSS, n=5) are shown below for comparison.

Supplementary Figure 4. Histology of ex vivo human liver slice cultures treated or not with CITCO. Upper images: HE stains of vehicle-treated control slices show examples of liver with well-preserved histology in the absence of treatment. Note the absence of regular hepatocyte architecture and/or the paucity of well-formed nuclei. Lower images: HE stains of CITCO-treated liver slices show one example of liver with ill-preserved histology (left) and one with well-preserved histology (right). Note the more regular liver architecture and hepatocyte nuclear structure in CITCO-treated versus vehicle-

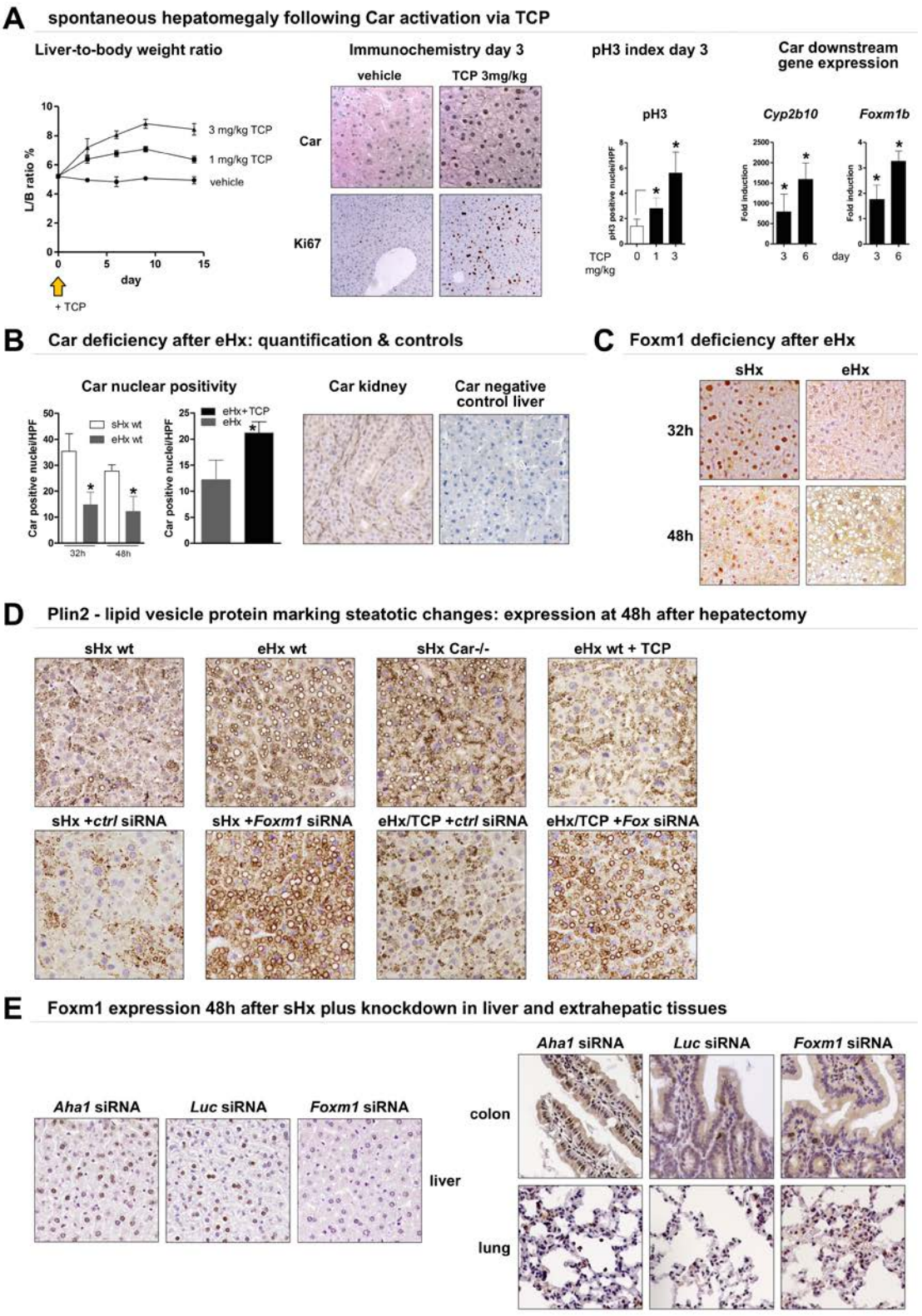
treated slices. Further note that unlike for CITCO-treated liver, ill-preserved histology dominated over well-preserved histology in vehicle-treated slices.

Supplementary Figure 5. Delayed TCP injection (after eHx) maintains the beneficial effects of concomitant injection (with eHx). Survival is shown after extreme (91%) hepatectomy normally leading to 100% mortality. TCP injection 3h or 9h following hepatectomy rescues 40% of mice, akin to concomitant injection (Fig. 1*D*). N=5/group; **P*<.05.

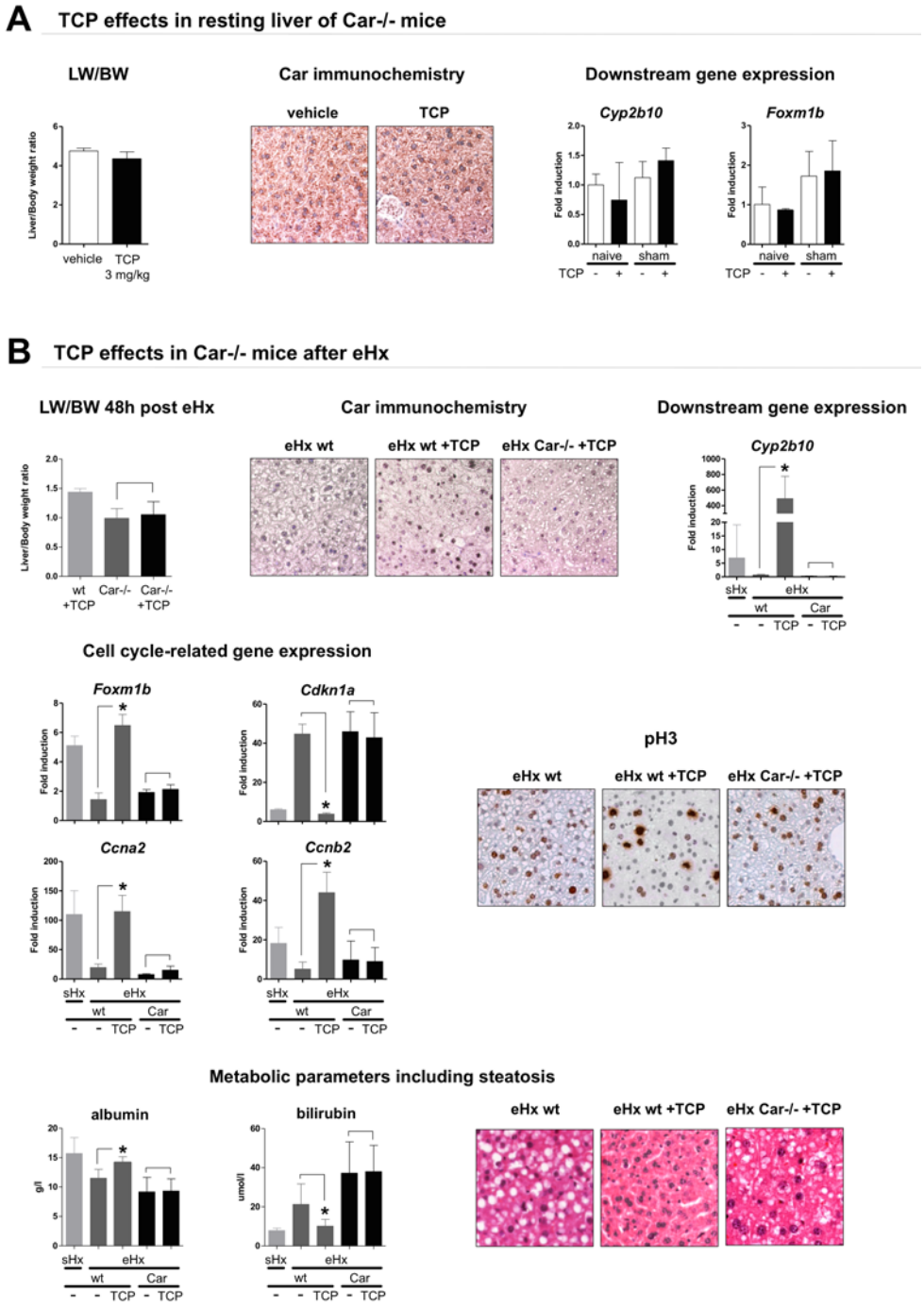
Supplementary Figure 6. CAR is downregulated in the majority of human HCC. CAR immunohistochemistry was performed on tissue arrays including formalin-fixed paraffin-embedded biopsies from 100 HCC and 90 non-malignant liver controls. Representative stainings are shown to the left. Less than 40% of HCC examined displayed nuclear or cytoplasmic CAR expression. CAR activation for the treatment of SFSS may be amenable to patients undergoing hepatectomies for HCC with downregulated CAR.

Supplementary Figures

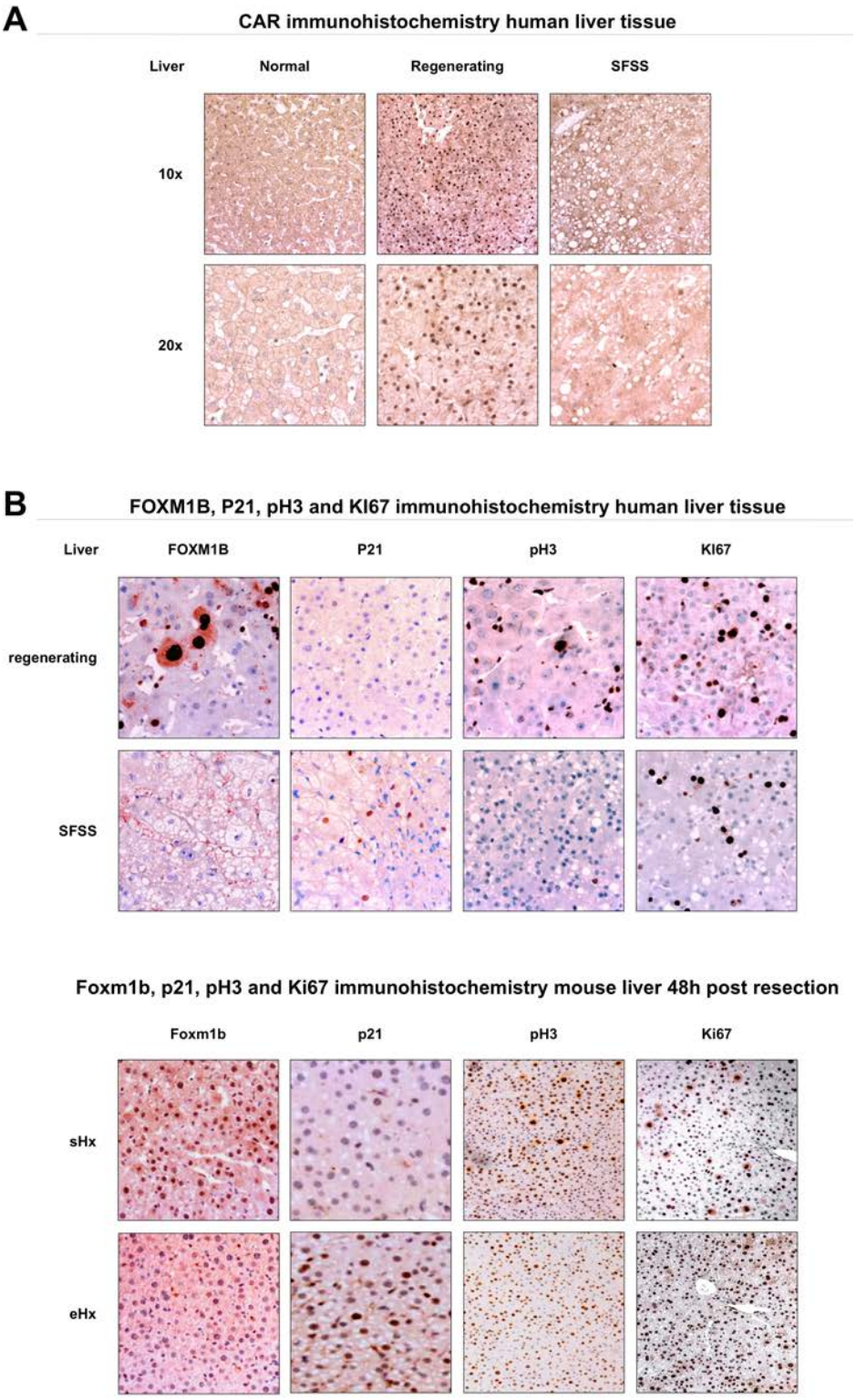
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



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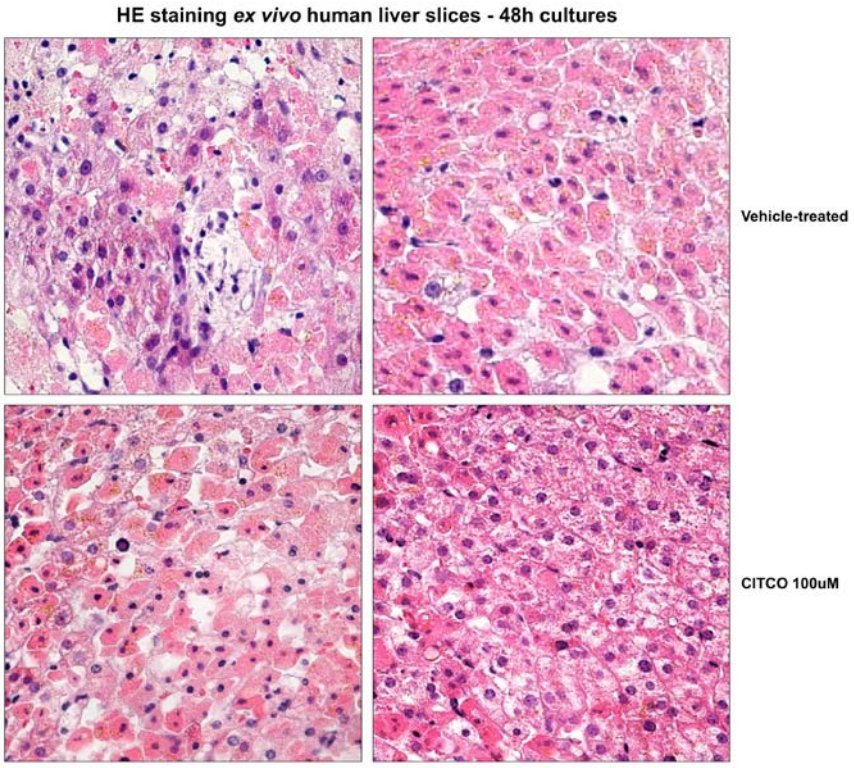
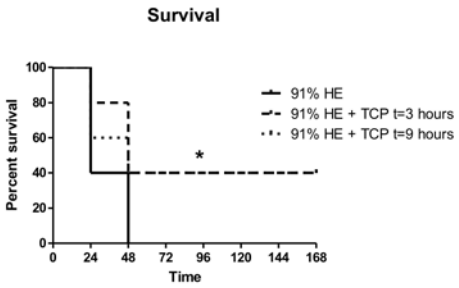
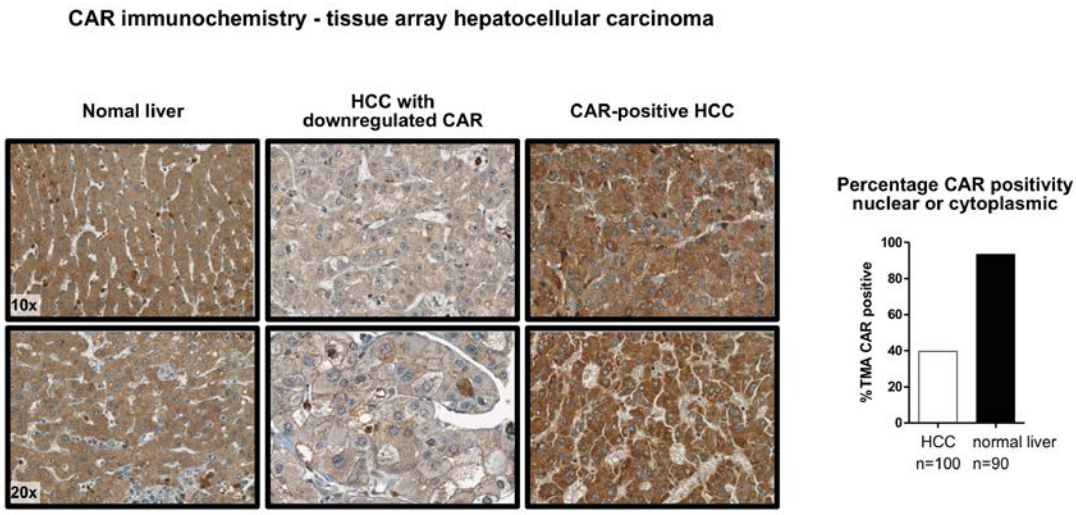


Supplementary Figure 5



Supplementary Figure 6



5. Manuscript B

PTEN downregulation promotes β-oxidation to fuel hypertrophic liver growth after hepatectomy in mice

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Contribution: This study represents main part of my PhD work. Most of the experiments were done by me solely and a few in collaboration. I strongly contributed to the design of the experiments and data interpretation. I participated in drafting of the manuscript as well.

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Abbreviations: AUC - area under the curve, LR – liver regeneration, Lw/Bw – liver weight to body weight ratio, PH – partial hepatectomy, RAS – regeneration associated steatosis, RER - respiratory exchange ratio, SFSS - small-for-size syndrome, TG - triglycerides

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Abstract

BACKGROUND & AIMS: In regenerating liver, hepatocytes transiently accumulate lipids before the major wave of parenchymal growth. This regeneration-associated steatosis (RAS) is required for liver recovery, but its purpose is unclear. The tumor suppressor PTEN is a key inhibitor of the AKT-mTOR axis, which regulates growth and metabolic adaptations after hepatectomy. In quiescent liver, PTEN causes pathological steatosis when lost, while its role in regenerating liver remains unknown. Here, we explored whether PTEN is linking RAS with growth during liver regeneration.

METHODS: We studied regeneration after partial hepatectomy in wild type mice, and in liver-specific *Pten*$^{-/-}$ mice shortly after induction of knockout. Proliferation, hypertrophy, and parameters related to glucose and lipid metabolism were assessed. Liver function was tested in a model of resection-induced liver failure. Energy substrate utilization was determined by indirect calorimetry, while the role of β-oxidation was probed through CPT1 inhibition.

RESULTS: In wild type mice, PTEN was downregulated after hepatectomy and associated with RAS turnover and hypertrophy. *Pten* knockout accelerated hypertrophic regeneration, resulting in hepatomegaly and raising survival after lethal resection. Following hepatectomy, the shift from glucose to lipid usage was enhanced by PTEN loss and correlated with the disappearance of RAS. Mild inhibition of β-oxidation led to persisting RAS in *Pten*-deficient mice and abrogated hypertrophic liver growth, which was rapamycin-sensitive.

CONCLUSIONS: PTEN downregulation after hepatectomy promotes the catabolism of RAS-derived lipids to fuel hypertrophic liver growth. These findings identify RAS as a provider of regenerative energy, emphasizing the need of an adequate lipid supply for successful liver regeneration.

KEYWORDS: respiratory exchange ratio; peripheral fat mobilization; small-for-size-syndrome

Introduction

Liver regrowth after tissue loss requires the orchestrated regeneration of all liver-resident cells. The functional units of liver, the hepatocytes, are regenerated first via both hyperplastic and hypertrophic mechanisms, followed by the reconstitution of non-parenchymal cells such as the liver sinusoids.[1,2] Regeneration is highly efficient, with removal of 70% of the liver leading to complete regrowth within a week in mice.[3] The enormous growth rate points to the need for suitable energy sources that fuel liver regeneration (LR).

Systemic metabolic changes after partial hepatectomy (PH) are thought to provide regenerative triggers, but might also serve to satisfy energy demands. Liver is the major glucose provider, and hypoglycemia inevitably develops when liver mass is lost. Indeed, hypoglycemia is an essential stimulus to induce regeneration.[1] Moreover, hypoglycemia is thought to trigger a systemic response leading to a redistribution of lipids from the periphery into the regenerating liver.[4] Unlike pathological steatosis (i.e. fatty liver disease), hepatocellular lipid accumulation after hepatectomy is a universal, physiological process that accompanies successful LR.[4] In mouse, regeneration-associated steatosis (RAS) peaks 16h after PH and then gradually declines to lean values by 48-72h, thus around the major wave of parenchymal growth.[3] RAS is needed for regeneration, because its disruption - be it through inhibition of peripheral fat mobilization or of fat droplet formation - impairs liver recovery.[5-7] Although first described more than 60 years ago,[8] the function of RAS remains unknown.

The tumor suppressor PTEN is a key inhibitor of the growth-promoting PI3K-AKT-mTOR axis. Specifically, PTEN opposes the phosphoinositide-dependent activation of AKT-mTOR through PI3K. Signaling through this pathway has profound impact on fundamental biological processes. The AKT-mTOR axis is considered a key regulator of cell-autonomous and systemic metabolism, orchestrating the metabolic and energetic needs with cellular growth also in response to altered states.[9] By opposing AKT-mTOR activation, PTEN holds a powerful position, and even minor reductions in its activity can have serious consequences.[10] The loss of PTEN in hepatocytes promotes lipid import and lipogenesis, rapidly leading to pathological steatosis, which then progresses to hepatitis and eventually cancer.[11-13] The PI3K-AKT-mTOR pathway does participate in LR, with interruption of this axis compromising recovery after hepatectomy. Intriguingly, PI3K seems to promote hyperplasia by phosphorylating STAT3, while the phosphoinositide-dependent AKT-mTOR axis stimulates hypertrophic growth.[1,14] Notably, AKT signaling regulates many of the metabolic adaptations associated with regeneration after tissue loss.[15] No direct evidence supports a role for PTEN in liver regeneration thus far; however the pro-regenerative functions of several miRNAs have been associated with the suppression of PTEN after resection.[16-18]

The downregulation of PTEN[16-18] suggests the phosphatase is liberating PI3K-mediated AKT-mTOR activities during LR. As such, PTEN reductions might regulate not only parenchymal growth, but also adaptations to altered metabolic demands following tissue loss. Given the steatotic phenotype of resting liver lacking PTEN,[11,12] RAS observed in regenerating liver might be related to the reductions in this tumor suppressor after hepatectomy. Furthermore, recent evidence indicates also catabolic roles for AKT-mTOR activities in hepatic lipid metabolism,[19] perhaps implying that PTEN contributes to the turnover of RAS. Therefore, downregulation of PTEN after hepatectomy might serve to orchestrate tissue growth with the resulting energy demands. To this end, we explored PTEN and associated changes in regenerating liver. More specifically, we aimed at identifying the role of PTEN in liver growth and RAS, with the goal to shed light onto the function of RAS after tissue loss.

Materials & Methods

Animals

Animals aged 8–10 weeks were kept on a 12h-day/night cycle with free food/water access. Male wild-type (wt) mice (C57BL/6) were from (Envigo, Horst, NL). Hepatocyte-specific inducible *Pten* knockout (PtenKO) animals (*AlbCre-ERT2$^{Tg/+}$Pten$^{fl/fl}$*) and corresponding controls (*AlbCre-ERT2$^{+/+}$Pten$^{fl/fl}$*, PtenC) were kindly provided by M. Foti (University of Geneva). PtenKO/PtenC breeding was started with offspring from in-house C57BL/6 following embryo transfer. Knockout was induced by tamoxifen (Sigma, 100ul, 20mg/ml in corn oil i.p. once a day for 5 consequent days) and animals were operated 4 days later. All animal experiments were in accordance with Swiss Federal Animal Regulations and approved by the Veterinary Office of Zürich.

Animal surgery and substances

Partial hepatectomy (68%, PH) and 91% hepatectomy (a model of lethal liver failure) were performed as reported.[20] Sham operation consisted of cholecystectomy. The gain in liver weight was expressed through the liver-weight-to-body-weight ratio (Lw/Bw). Wortmannin (in 10%DMSO), etomoxir (in H_20) and rapamycin (10%DMSO) were from Sigma (Buchs, Switzerland), while bpV (in saline) was from Merck Millipore (Darmstadt, Deutschland). Substances were i.p. injected in 100µl volume at doses and times indicated in the results.

Histological staining

H&E, PAS and immunochemical stainings were performed on 3µm archived liver sections and Oil Red O on cryosections. Antibodies used are listed in Supplementary Table 1; immunochemistry was performed with a Dako Autostainer Link48 Instrument and the iView DAB kit (Dako Glostrup, Denmark).[3,20] Quantification of Ki67- and pH3-positive hepatocytes was done by blinded manual counting in 10 random visual fields (20x).

Hepatocyte size

The ratio of cytoplasmic area/number of hepatocyte nuclei was histologically assessed on H&E images taken randomly at 40x magnification (5 images/sample) in a blinded way. Threshold was applied for area quantification (ImageJ NIH software) to exclude vessels and lipid droplets. Forward scatter was used to measure cell size in a flow cytometer BD FACSCantoII (BD Biosciences, Eysins, Switzerland) via FACSDiva v6.1.2 software. Hepatocytes were isolated from liver following perfusion and density centrifugation as described (http://munin.uit.no/bitstream/handle/10037/4575/article.pdf?sequence=1).

Western blotting

The procedure was performed as reported[3,20] with antibodies listed in Supplementary Table 1.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from 50 mg of tissue using Trizol reagent (Invitrogen, Basel, Switzerland). qPCR was performed on cDNA (Thermo Script reverse transcription PCR System, Invitrogen) using the ABI Prism 7500 Sequence Detector System (PE Applied Biosystems, Rotkreuz, Switzerland) as described.[3,20] 18S *rRNA*-normalized expression values were presented as fold induction ($2^{-\Delta Ct}$) relative to time-matched sham samples, and relative to uninduced liver for PtenKO/C. Taqman gene expression assays (Applied Biosytems) are listed in Supplementary Table 2.

Indirect calorimetry and Echo-MRI

Indirect calorimetry experiments were conducted at the Small Animal Metabolic Phenotyping core facility (University of Geneva), and approved by the Geneva Health Head Office. Energy expenditure and the respiratory exchange ratio (RER) were derived from O_2 consumption and CO_2 production; RER differences between PtenKO and PtenC were assessed through AUC (area-under-the-curve) analysis. Locomotor activity was recorded by an infrared frame, and food/fluid intake were measured by respective sensors. Parameters were recorded in mice individually housed in Labmaster metabolic cages (TSE, Bad Homburg, Germany) after 5 days of adaptation. After recording day 2, mice were operated, underwent Echo-MRI and were returned to metabolic cages for another 3 recording days. An EchoMRI-700 quantitative nuclear magnetic resonance analyzer (Echo Medical Systems, Houston, TX) was used to measure total fat and lean mass.

Lipid and glycogen measurements

Quantitation kits were used to measure triglycerides (Abcam, Cambridge, UK; ab65336). HDL (Sigma, Buchs, Switzerland; MAK045-1KT), and glycogen (Sigma; MAK016-1KT).

Statistical analysis

Data are presented as mean ±SD unless stated otherwise. Differences between groups were assessed by a two-tailed t test assuming unequal variance. In general, $n \geq 5$ mice/group were analyzed. For survival after 91%-hepatectomy, 10 animals/group were included. Differences were considered significant at $p<0.05$ and indicated by an asterisk (∗). Statistical analyzes were performed using Prism 6.0 (GraphPad).

Results

PTEN is associated with RAS turnover, weight gain and hypertrophy in regenerating liver

To explore the roles of PTEN and RAS, we assessed lipid-associated parameters and PTEN levels following standard (68%) hepatectomy (PH) in wt mice. Histology confirmed the RAS peak at 16h and its gradual disappearance around the times of major parenchymal growth.[3,8] The steatotic peak was preceded by the induction of *Plin2*, which promotes hepatocyte-adipocyte transdifferentiation and is required for lipid droplet formation.[21] Around the steatotic peak, *Cd36* (fatty acid translocase) was upregulated, consistent with peripheral import of fat.[6] Further, β-oxidation genes (*Cpt1a*, *Hadha/b*) were

elevated at the expense of lipogenic genes (*Scd1a*, *Acc*, *Fasn*) (Fig. 1B), suggesting fat is being accumulated for energetic needs.

Significant PTEN protein downregulation (Fig. 1C) occurred at the RAS peak, but after *Plin2* induction, and persisted during lipid disappearance. PTEN reduction may hence be associated with RAS turnover but not with its accumulation.

To estimate PTEN's function during LR, we simulated elevated PTEN activity through the inhibition of PI3K. Injection of wortmannin (0.75mg/kg) at 13h post PH led to reduced mitoses, a reduced hepatocyte size (however only if lipid vesicles were excluded from hepatocyte area), but an increased liver-to-body-weight-ratio (Lw/Bw) at 48h. The latter was likely due to lipid accumulation, which was strongly elevated compared to vehicle controls (Fig. 1D).

When PTEN was inhibited by bpV (3.3mg/kg) at 13h post PH, Lw/Bw and hepatocyte size were increased, while RAS was diminished (Fig. 1E). Notably, mitotic counts were also reduced through bpV, suggesting that PTEN inhibition during LR specifically affects the phosphoinositide-*dependent* AKT-mTOR axis, which promotes hypertrophy at the expense of hyperplasia.[14] In contrast, PI3K inhibition additionally affects phosphoinositide-*independent* STAT3 activation, hence impacting on both hyperplasia and hypertrophy.[14] Taken together, these findings indicate PTEN downregulation is associated with RAS turnover, weight regain, and hypertrophy in regenerating liver.

Hepatocyte-specific Pten deficiency accelerates functional liver recovery via hypertrophy

PTEN inhibition after hepatectomy promotes liver weight recovery, however bpV acts systemically and may exert unspecific effects. We therefore used inducible hepatocyte-specific *Pten* knockout mice to define the impact of PTEN deficiency on LR. Knockout was induced by TAM in *AlbCreERT2*$^{tg/+}$-*Pten*$^{fl/fl}$ (PtenKO) 4d prior to hepatectomy to avoid pre-existing fatty liver that may impair regenerative capacity.[22] *AlbCreERT2*$^{+/+}$-*Pten*$^{fl/fl}$ lacking Cre served as controls (PtenC). *Pten* expression was not significantly altered after PH in PtenC (Fig. 2A), suggesting PTEN downregulation (Fig. 1C) is regulated posttranscriptionally following resection. Regenerated livers in PtenKO mice remained *Pten* deficient, reflecting liver reconstitution from differentiated (albumin-positive) hepatocytes (Fig. 2A).

At PH, the starting weight of the liver remnant was slightly increased in PtenKO relative to PtenC. After PH, the difference in liver weight became more pronounced over time, leading to hepatomegaly in PtenKO after a week (Fig. 2A). Notably, accelerated weight gain was not

associated with proliferation (Fig. 2B; Suppl. Fig. 1), but with enhanced hepatocellular hypertrophy (Fig. 2C).

To determine whether accelerated weight gain leads to improved liver recovery, we performed 91%-hepatectomy, which causes lethal liver failure in wt mice.[20] The best measure to assess recovery of liver function is seven-day-survival, the critical period after liver loss. Remarkably, survival was raised to 40% after 91%-resection in PtenKO mice (Fig. 2D), indicating that the surplus hypertrophic liver mass generated by PTEN deficiency is functional.

Given the significant hypertrophy at 72h post PH in PtenKO, we investigated AKT-mTOR-S6K signaling, known to promote a hyperplasia-to-hypertrophy switch.[14] Activating AKT phosphorylation was markedly increased by PTEN deficiency (Fig. 2E). Likewise, enhanced S6K phosphorylation indicated elevated mTORC1 activity. Increased phosphorylation of AKT and S6K was also evident at 32h (Suppl. Fig. 2), consistent with AKT-mTORC1-S6K signaling as a hypertrophic driver in regenerating liver from PtenKO.

Pten deficiency promotes glucose storage and lipid metabolism in regenerating liver

Acceleration of liver regeneration in PtenKO is expected to rely on additional energy supply. We hence assessed hepatic energy stores, mainly consisting of triglycerides (TGs) and glycogen.

Following hepatectomy, both PtenKO and PtenC displayed early drops in liver glycogen and serum glucose (Fig. 3A), a reported signal required for the initiation of regeneration.[4] Notably, glycogen stores recovered faster in PtenKO relative to PtenC, accompanied by downregulation of gluconeogenic expression (*Ppargc1a*, *Pck1*, *G6pc*; Fig. 3A). Given the similar serum glucose levels, these findings indicate PTEN deficiency counteracts glucose usage in regenerating liver.

Hepatic TG content was elevated in PtenKO at hepatectomy and peaked at 16h akin to PtenC (Fig. 3B; see Supplementary Fig. 3 for chemical fat analysis). The subsequent decline in TG levels was delayed in PtenKO relative to PtenC. Therefore, the loss of PTEN prior to hepatectomy leads to an expanded RAS period. In contrast, inhibition of PTEN at the RAS peak shortened this period (Fig. 1E), suggesting PTEN deficiency does not directly enhance RAS formation during LR. An expanded RAS period may hence be secondary to the increased hepatoperipheral lipid shuttle pre-existing in PtenKO before PH.[11] Accordingly, serum TGs were elevated in PtenKO at PH, but dropped during the RAS peak similar to PtenC (Fig. 3B). Likewise, serum levels of HDL - transporting TGs into liver - were increased in PtenKO versus PtenC during LR (Fig. 3B).

Moreover, the expression of *Lipe* and *Lpl* - lipases that free fatty acids from TGs[23,24] - was elevated in PtenKO (Fig. 3B). On the other hand, *Plin2* and *Cd36* expression was unaffected by PTEN knockout (Supplementary Fig. 3), again suggesting PTEN deficiency does not directly promote RAS formation in regenerating liver (Fig. 1A/C). Accordingly, expression of *Fabp4* - required for hepatocyte-adipocyte transdifferentiation and upregulated in resting PtenKO liver[12] - was not enhanced and even downregulated by PTEN loss during the RAS period (Supplementary Fig. 3).

The changes in lipid content further suggest that hypertrophy in PtenKO is not due to fat elevations, because at 72h the gains in hypertrophy and Lw/Bw were marked (Fig. 2A/C) despite a minimal lipid content (Fig. 3B, Supplementary Fig. 3). Hypertrophy may hence relate to the elevated glycogen content (Fig. 3A) in regenerating PtenKO liver.

Besides peripheral fat import, resting PtenKO liver is known to accumulate fat also via increased lipid synthesis.[11] Accordingly, lipogenic molecules (SREBP1 and its transcriptional targets *Scd1*, *Fasn*) were upregulated at hepatectomy in PtenKO liver remnants (Fig. 3C). After hepatectomy, however, the lipogenic program was suppressed (Fig. 3C), indicating little contribution of hepatic lipogenesis to RAS.

Finally, we assessed the expression of *Fgf21*, a catabolic molecule shown in the liver to inhibit lipogenesis but promote glycogenesis and lipid oxidation.[25,26] In both PtenKO and PtenC, *Fgf21* expression peaked with RAS. In PtenKO, a

second peak was present around 48h post PH (Fig. 3D), implying a prolonged catabolic phase perhaps related to the extended period of RAS and its disappearance at 72h in the mutant animals.

Altogether, these results suggest that the expanded RAS period in PtenKO results neither from increased lipogenesis nor from elevations in active lipid import. Rather, the systemic redistribution of lipids into liver after tissue loss[4,27] may be enhanced due to an elevated mobilization of peripheral fats pre-existing in PtenKO liver before PH,[11,12] and perhaps because of a higher catabolism of lipids within the liver.

Pten deficiency promotes lipid oxidation as an energy source after hepatectomy

To gain insight into concrete metabolic outputs during LR and the effects of PTEN loss thereupon, we measured RER (respiratory exchange ratio CO_2/O_2) after PH via indirect calorimetry. The typical diurnal shift towards lipid oxidation was recorded for both PtenKO and PtenC (Fig. 4; see Supplementary Fig. 4 for control data). On resection, RER markedly dropped during the first 32h, identifying lipid oxidation as the favored energy source during the RAS period of regenerating liver. Unlike for PtenC, RER around the RAS peak remained close to 0.7 in PtenKO, indicating pronounced fat catabolism when PTEN is low. From 32h onwards, RER began to rise towards pre-hepatectomy levels. Another reduction in RER was recorded in the mutant animals towards 72h, thus before TGs were disappearing in PtenKO liver. Therefore, LR leads to a profound increase in global lipid oxidation, which is enhanced through hepatic PTEN downregulation and correlates with the disappearance of liver fat.

Pten deficiency fuels hypertrophic LR via β-oxidation of RAS-derived lipids

The increases in lipid oxidation seen in PtenKO animals after hepatectomy may originate from the metabolism of RAS lipids to fuel the regenerative process. When assessing *Fabp2*, needed for import of fatty acids into and their oxidation within mitochondria, its expression was consistently increased in regenerating PtenKO compared to PtenC liver. Moreover, CPT1A, the key β-oxidation enzyme, was upregulated at the RAS peak in PtenKO liver (Fig. 5A).

If RAS lipids are oxidized in mitochondria to provide energy for liver growth, inhibition of β-oxidation should result in persisting steatosis and a diminished liver weight after hepatectomy. We first tested *siRNA* knockdown of *Cpt1a* using a formulation preferentially targeting hepatocytes.[20] While efficient *Cpt1a* knockdown was achieved in liver, CPT1A protein was not affected (data not shown), perhaps owing to a long half-life ongoing with its vital function.[28] Instead we treated animals with the CPT1 inhibitor etomoxir. High doses (20mg/kg) given 16h after resection led to rapid death of animals, likely due to the systemic action of etomoxir on all CPT1 isoforms in tissues such as the heart. When applying lower doses (10mg/kg) every 12h starting at 16h post PH, mice survived and tissue could be analyzed. At 72h after PH - when liver is lean and PtenKO display hypertrophy with elevated liver weight - etomoxir did not significantly alter Lw/Bw or hepatocyte size in PtenC relative to vehicle controls, despite a mild increase in hepatic lipids (Fig. 5B). The lack of distinct effects may not only relate to the low dose but also to the fact that etomoxir will not inhibit the oxidation of medium and short chain fatty acids. In PtenKO however, etomoxir reduced liver weight while markedly increasing the hepatic TG content. Moreover, hepatocyte size of PtenKO was diminished by etomoxir and no more different from that of PtenC hepatocytes (Fig. 5B). Therefore, mild inhibition of β-oxidation does counteract the hypertrophic parenchymal growth driven by PTEN loss, however it appears insufficient to impact on the general regeneration process. We conclude that RAS provides lipids for β-oxidation to fuel hypertrophic LR, a process promoted by PTEN downregulation.

mTOR mediates PTEN loss-driven hypertrophy in regenerating liver

Both AKT and mTORC1-S6K have been implicated in the regulation of hepatocellular hypertrophy.[4] The activities of both AKT and

mTORC1-S6K were elevated in PtenKO relative to controls (Fig. 2E, Supplementary Fig. 2). To reveal contributions of these molecules to the processes driven by PTEN loss, we treated PtenKO and PtenC with moderate doses of the specific mTORC1 inhibitor rapamycin, injecting 1mg/kg at 13h post PH and subsequently every 24h until harvest. In PtenC at 72h post resection, rapamycin had little effect on liver weight, the reduction of hepatocyte size, and the hepatic TG content relative to vehicle controls (Fig. 6). In PtenKO, rapamycin significantly reduced liver weight and hepatocyte size, but did not significantly impact on TG content. These findings portray mTOR is a major promoter of hypertrophy in *Pten* loss-driven regeneration, while lipid metabolism may preferably be regulated also via mTOR-independent paths in regenerating liver.

Discussion

Transient steatosis occurs in every regenerating liver and meanwhile is recognized as an essential component of successful recovery after tissue loss. The precise function of RAS has remained unknown thus far, however the delivery of energy for the regenerative process provides a conceivable explanation. Here, we show that the tumor suppressor PTEN participates in liver regeneration (LR), with its downregulation promoting liver growth fueled by RAS. More specifically, we demonstrate that (i) RAS turnover is associated with the downregulation of PTEN after PH; (ii) PTEN deficiency promotes AKT-mTORC1 signaling and hypertrophic liver growth; and (iii) β-oxidation of RAS-derived lipids is required for hypertrophy driven by PTEN deficiency. Moreover, our data indicate a general shift to lipid oxidation during LR, consistent with the view that the burning of fat is a main energy source for the regenerative process.

Following PH, PTEN is downregulated at a time when lipid accumulation peaks in regenerating liver. Our initial experiments using wortmannin and bpV suggested PTEN regulates hypertrophic liver growth in association with RAS turnover. To better appreciate the function of PTEN downregulation, we explored regeneration in liver with hepatocyte-specific PTEN loss.

In quiescent liver, the loss of PTEN causes pathological steatosis developing from elevated lipogenesis and an increased import of peripheral lipids, likely related to exaggerated insulin-PI3K-AKT signaling.[11-13] In regenerating liver, *Pten* loss had a different outcome. Although *Pten* loss was associated with an expanded RAS period, it had no impact on molecules that are upregulated in resting PtenKO liver to promote lipid accumulation (i.e. import, vesicle formation, lipogenesis) - in keeping with PTEN's role in normal regenerating liver, where RAS formation occurred prior to PTEN downregulation. These observations are remarkable inasmuch as they illustrate how after tissue loss the regenerative program dominates over other processes, adapting the function of PTEN (and likely other molecules) for its own purposes.

While PTEN deficiency in resting liver mainly promotes energy storage, our studies on regenerating liver reveal a catabolic function for PTEN downregulation. Calorimetric measurements demonstrated a clear shift towards lipid consumption during the RAS period in PtenC. This shift was accentuated in PtenKO and preceded the disappearance of hepatic TGs after PH. Lipid oxidation was accompanied by an elevation in β-oxidation molecules and a reduction in glucose utilization in regenerating PtenKO liver, emphasizing fat as a preferred energy source. Therefore, PTEN downregulation after hepatectomy appears to foster a unique phenotype in that it promotes lipid oxidation while enhancing glucose storage.

Although mild inhibition of long chain fatty acid oxidation had no effect on liver growth in wt mice, the marked calorimetric shift towards lipid oxidation after PH also in PtenC - together with the upregulation of β-oxidation genes in wt mice - strongly suggests that RAS serves as a general provider of energy for regenerating liver. Previous research is fully consistent with this view; in 2004, Shteyer *et al.* showed that the inhibition of RAS through either leptin or the deletion of hepatic glucocorticoid receptors suppresses regeneration after PH.[5] Although the

relevance of RAS was questioned in a subsequent study,[29] mounting evidence from various approaches consistently points to RAS as crucial for successful regeneration. Accordingly, counteracting peripheral fat mobilization prior to PH is sufficient to prevent RAS and to inhibit regeneration, with depletion of peripheral fat stores being proportional to the lost volume.[6,7] Besides interfering with peripheral fat import, deficient regeneration is likewise induced through a variety of other measures that counteract RAS formation, including β-glucosylceramide pretreatment,[30] knockout of *Plin2* (impaired lipid vesicle formation),[21] or *Tm7sf2* deletion (defective cholesterol biosynthesis).[31] Additionally, animal models associated with deficient β-oxidation - such as due to knockout of adiponectin or other molecules regulating energy usage - present with deficient regeneration and persisting RAS.[32-35] Finally, earlier experiments with the β-oxidation inhibitor octanoylcarnitine pointed to lipids as the dominant energy source early after PH.[36]

Importantly, in both models of PTEN deficiency, liver weight and hepatocyte size were increased at times when TG content was close to nil (i.e. bpV 48h, PtenKO 72h), linking PTEN deficiency, RAS turnover and hypertrophic liver growth. When β-oxidation was mildly inhibited from the RAS peak onwards, hepatic TGs remained elevated at 72h in PtenKO, ongoing with a reduction in liver weight and hepatocyte size. Overall, these findings identify RAS as a source of lipids that are being oxidized to fuel hypertrophic liver growth in a PTEN-dependent manner (Fig. 7).

Our observation of PTEN downregulation as a promoter of hypertrophic LR is not unexpected. PTEN deficiency occurs in various hypertrophic pathologies, and cell type-specific *Pten* loss may be etiological in diseases such as cerebellar hypertrophy,[37] macrocephaly,[38] or hypertrophic cardiomyopathy.[39] Notably, the hypertrophic changes in animal models of above diseases can be corrected by the inhibition of mTORC1.[37-39] Similarly, moderate dose rapamycin treatment of PtenKO during the RAS peak inhibited both hepatocellular hypertrophy and the excess liver weight gain, consistent with the PDK1-AKT-mTORC1 axis as a promoter of hypertrophic regeneration.[14] Whether rapamycin may specifically affect mTORC1 or also mTORC2 in liver is a matter of debate.[40,41] In any case, our observation is consistent with mTOR as a central regulator of cell size.[42] Recent findings indicate that mTORC1-S6K can promote *Cpt1a* expression and lipid oxidation in resting liver.[19] However, rapamycin did not increase TG content in regenerating liver, suggesting either incomplete inhibition or a metabolism of RAS-lipids by other molecules regulated through PTEN.

AKT is known to directly regulate hepatic lipid metabolism, however the outcomes appear to be context-dependent. Established is AKT's role in fostering lipid accumulation, via promoting SREBP-dependent lipogenesis[43] and via suppression of β-oxidation.[44,45] Conversely, AKT seems to promote lipid turnover in other situations, such as in ACE2-mediated amelioration of fatty liver disease.[46] The antisteatotic properties of exercise have also been related to the upregulation of AKT.[47] Perhaps most fascinating is the association of AKT activity with increased hepatic lipid oxidation and oxidative capacity triggered by calorie restriction.[48] Fasting provokes hypoglycemia, glycogen depletion, mobilization of peripheral fats and a switch to lipid usage,[49] thus processes that we also observe in regenerating liver. Intriguingly, hepatocyte-specific deletion of *Sirt1*, a key energy sensor activated through nutritional deprivation, causes deficient liver regeneration accompanied by persisting RAS and a failure to upregulate genes needed for lipid oxidation.[35] More so, insulin levels drop upon fasting, while in regenerating liver insulin responsiveness appears to be dampened.[50] In terms of energy changes, the instant events occurring after hepatic tissue loss may hence be comparable to the early response towards acute fasting, albeit coupled to the specific demands of a growing tissue. The reduced insulin responsiveness further suggests AKT activation after PH occurs independent of insulin, perhaps explaining the divergent outcomes (i.e. fat storage *versus* usage) of AKT-mTOR signaling in resting (with overactive

insulin signaling) *versus* regenerating PtenKO liver.

The contribution of hypertrophy to liver regeneration is being increasingly appreciated.[51,52] Indeed, the promotion of hypertrophic liver growth through PTEN downregulation might be of clinical relevance. The most frequent cause of death due to liver surgery is the small-for-size syndrome (SFSS). This clinical entity can develop following too extended resection, leaving behind marginal remnants that fail to recover due to deficient regeneration.[3,20] Intriguingly, a consistent feature of SFSS livers is persisting hypersteatosis, illustrating the intimate relationship between RAS and successful recovery. No treatment currently exists for this entity, however experimental approaches that prevent the SFSS also normalize RAS.[20] Indeed, PTEN knockout was able to rescue 40% of mice after 91% hepatectomy, a lethal mouse model of the SFSS.[20] Therefore, transient inhibition of PTEN - such as through bpV - may offer new avenues to prevent or treat the SFSS. Moreover, the promotion of lipid oxidation may aid such measures, as suggested by the impact of lipid-plus-carnitine infusion on regeneration in rats.[53]

In summary, our study highlights the function of PTEN in compensatory liver hypertrophy and emphasizes the importance of RAS for the regenerative process. Following PH, the reductions in PTEN have two major consequences, that is the promotion of cellular hypertrophy via mTOR along with the enhancement of β-oxidation - a process that appears to be independent of mTOR but provides the fuel for the hypertrophic expansion of functional parenchyme. The catabolic properties of PTEN downregulation enhance the turnover of RAS, which forms as a part of the metabolic adaptations imposed by the loss of liver tissue. More broadly, our findings affirm RAS as an obligate component of successful liver regeneration, with fat as the prime regenerative fuel during the periods of low hepatic capacity. The appreciation of lipid catabolism in liver regeneration may point to novel options for the management of regenerative deficiencies in the clinic, such as experienced in surgery-induced liver failure.

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Author's contribution

Study concept & design: BH. Experimental design: EK, BH, MF. Data acquisition and analyses: EK, CT, NC, NB, PL, BH. Data interpretation: EK, BH, NC, MF, RG. Provision of preliminary data: ACP, JFD. Provision of transgenic mice: MF. Manuscript writing: BH, EK. Critical revision: BH, EK, RG, MF, JFD, PAC. Funding: BH, PAC.

Disclosures and conflict of interest statement: All authors contributing to this manuscript declare that they have nothing to disclose. They do not have a conflict of interest with respect to this manuscript.

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Figures

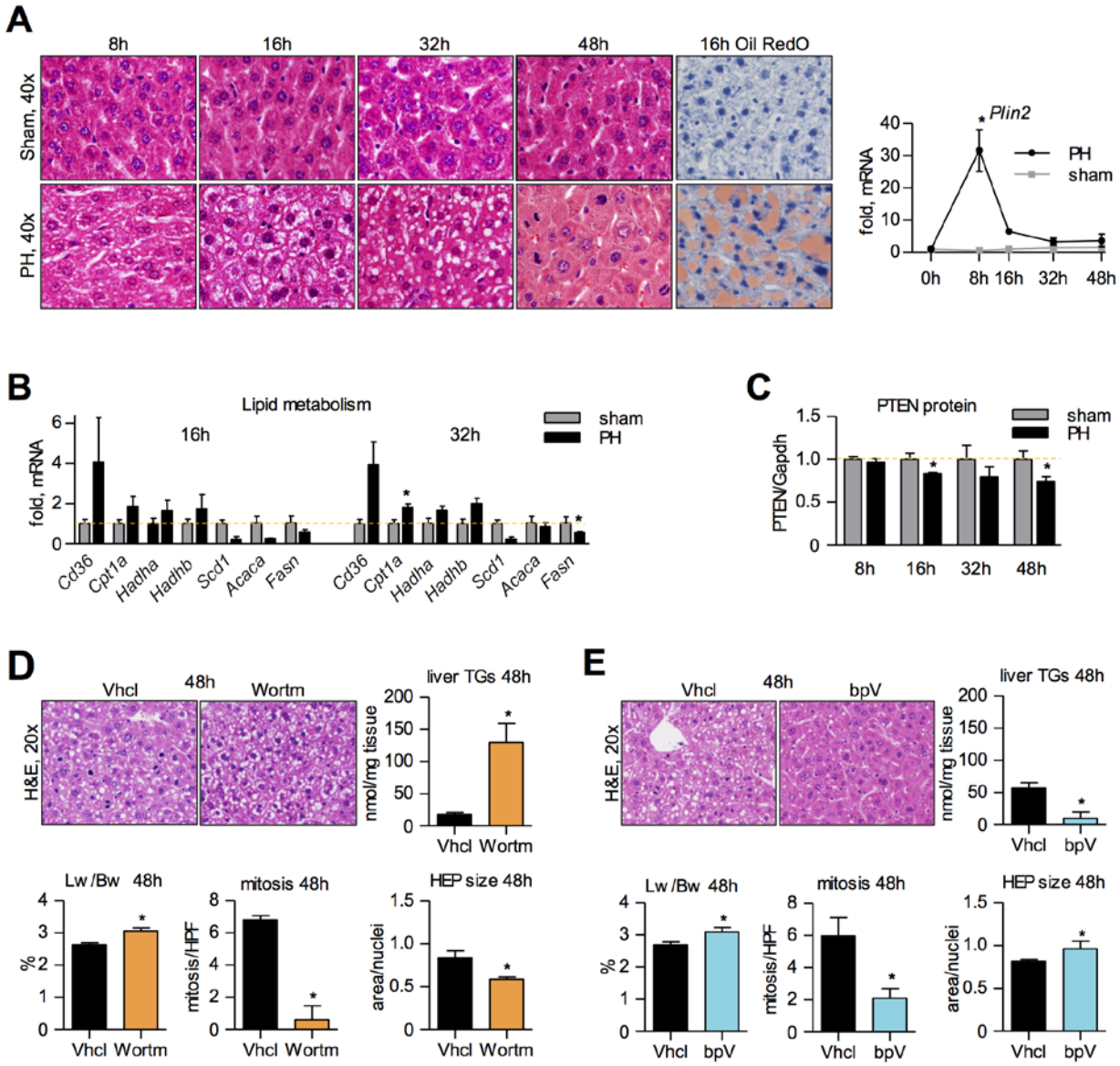

Figure 1. Association between RAS, PTEN and liver regeneration in wt mice. (*A*) Lipid accumulation (HE and Oil RedO stains) after sham surgery or resection. Hepatic *Plin2* expression is shown to the right. (*B*) Hepatic gene expression for *Cd36* (lipid import), *Cpt1a*, *Hadha*, *Hadhb* (β-oxidation), and *Scd1*, *Acaca*, *Fasn* (lipogenesis). (*C*) Quantified immunoblots for PTEN levels after surgery. (*D*) Wortmannin ("high" PTEN activity) and (*E*) bpV ("low" PTEN activity) effects on histological RAS, hepatic TGs, LWBW regain, mitoses and hepatocyte size at 48h after PH. n≥5/group for mean±SD; *P<0.05.

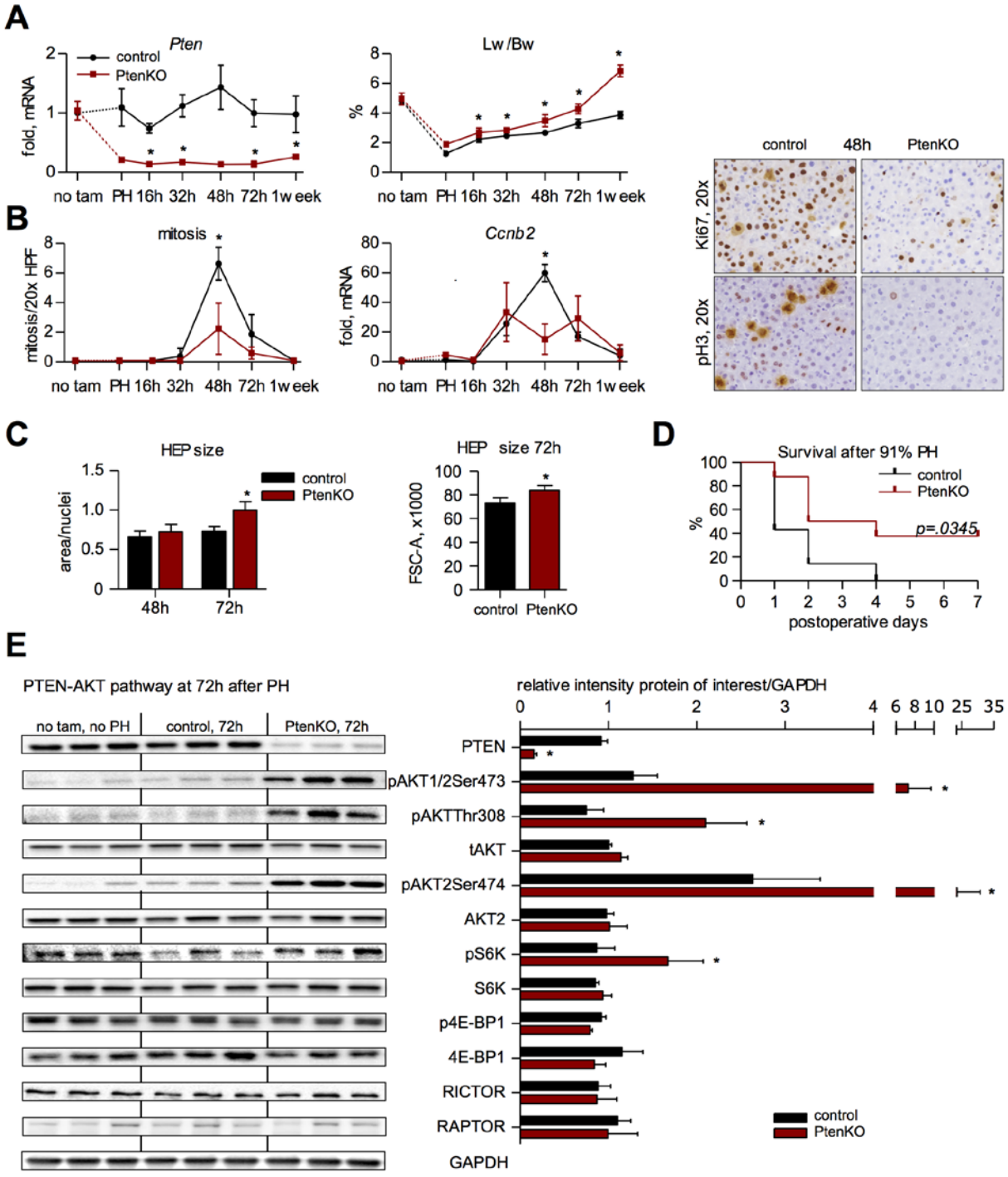

Figure 2. Promotion of liver regeneration through hepatic *Pten* loss. (*A*) *Pten* expression and Lw/Bw following tamoxifen-treatment and PH in PtenKO and PtenC (control) mice. (B) Mitotic counts and cyclin B2 gene expression. Ki67 and pH3 stains of PtenKO/PtenC liver post PH are shown to the right. See also Supplementary Fig. 1. (*C*) Hepatocyte size after resection on histology (left) and by cytometry (right). (*D*) Seven-day survival after lethal 91% PH in PtenKO and controls. (*E*) AKT-mTOR signaling in PtenKO/C assessed by immunoblots 72h after PH. n≥5/group for mean±SD; *P<0.05.

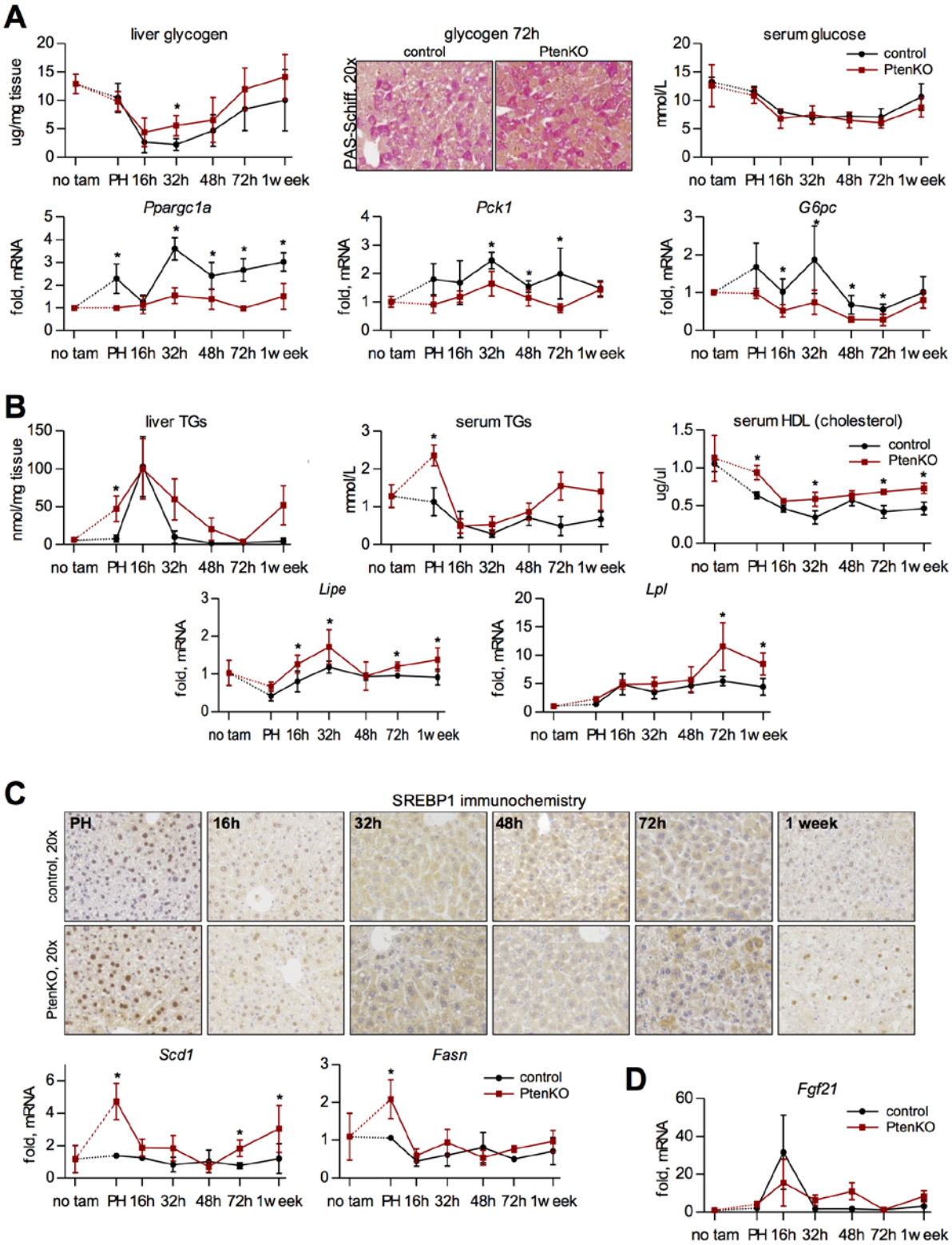

Figure 3. Glucose and lipid metabolism in PtenKO and PtenC after PH. (*A*) Hepatic glycogen content, serum glucose and hepatic expression of gluconeogenic genes (*Ppargc1a*, *Pck1*, *G6pc*). (*B*) Hepatic and serum TG levels, serum HDL, and hepatic expression of the genes encoding lipases LIPE and LPL. (*C*) Immunochemistry for the transcription factor SREBP1 and gene expression of its lipogenic targets *Scd1* and *Fasn*. (*D*) Hepatic gene expression of *Fgf21*. n≥5/group for mean±SD; *P<0.05.

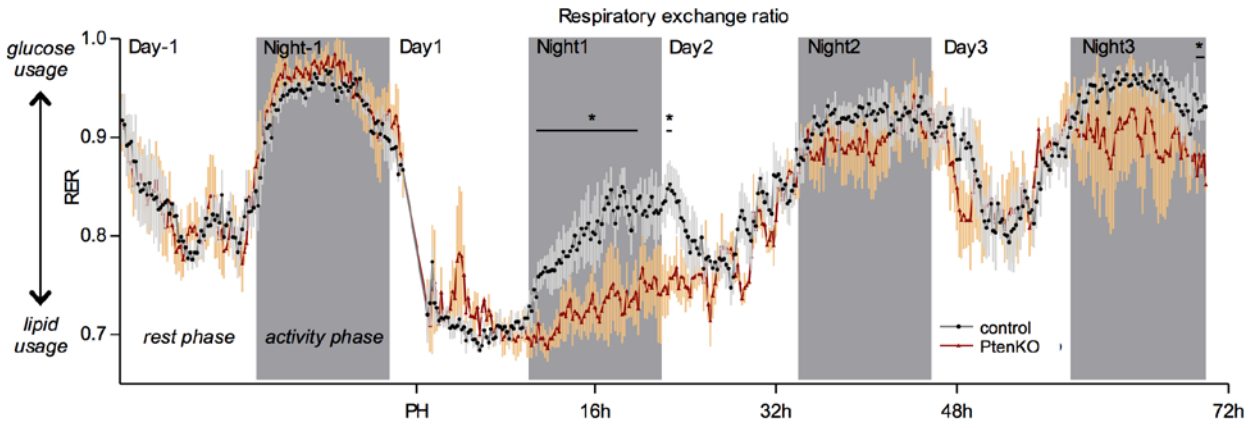

Figure 4. Bodily substrate usage in regenerating PtenKO and PtenC mice. Indirect calorimetry via metabolic cage measurements of RER before and after PH. n=5/group for mean ±SEM; *P<0.05.

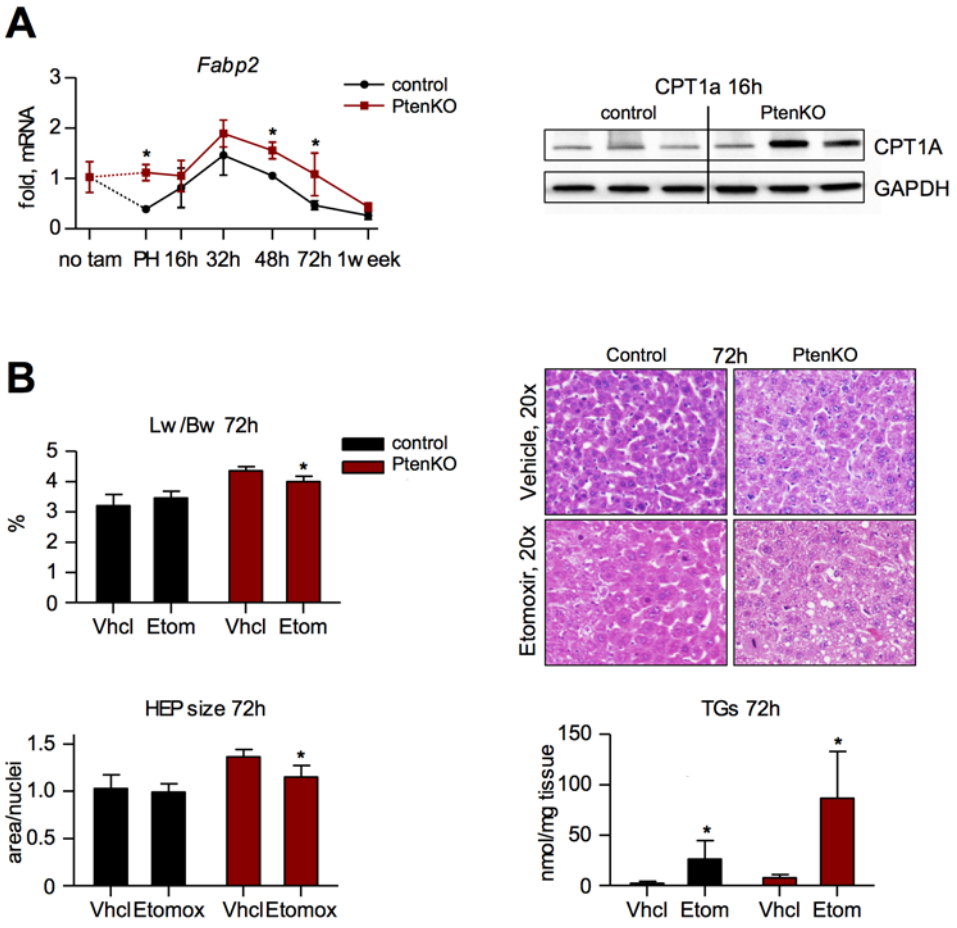

Figure 5. β-oxidation associates hypertrophy with RAS in regenerating PtenKO liver. (*A*) *Fabp2* gene expression and CPT1A protein expression in PtenKO/C at the RAS peak. (*B*) Impact of low dose etomoxir on Lw/Bw, hepatocyte size and RAS (histology, TG content) in PtenKO/C at 72h port PH. n≥5/group for mean±SD; *P<0.05.

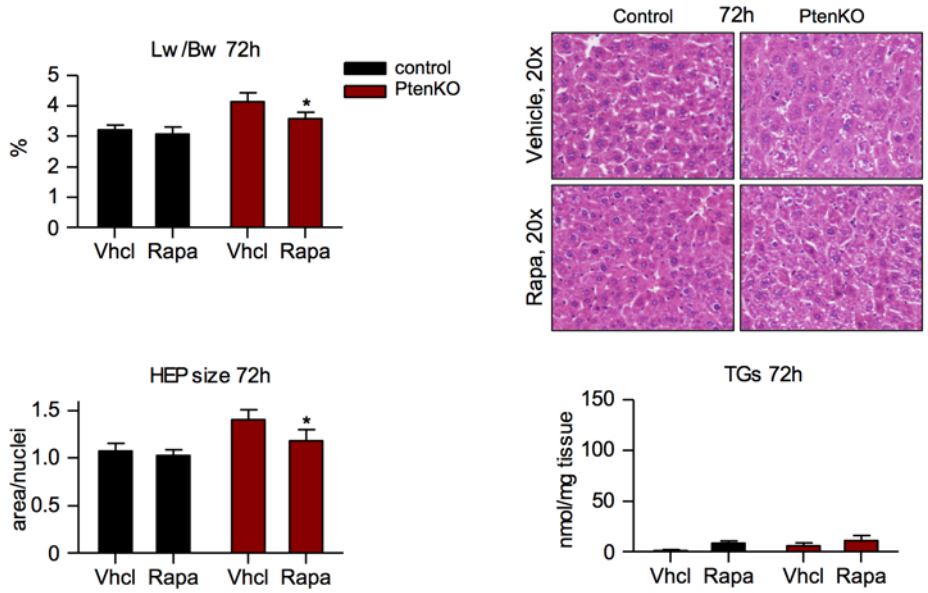

Figure 6. Dependency of hypertrophy but not lipid oxidation on mTOR in PtenKO. Effects of rapamycin treatment during RAS on Lw/Bw, hepatocyte size, and hepatic TG content in PtenKO and PtenC at 72h after PH. n≥5/group for mean±SD; *P<0.05.

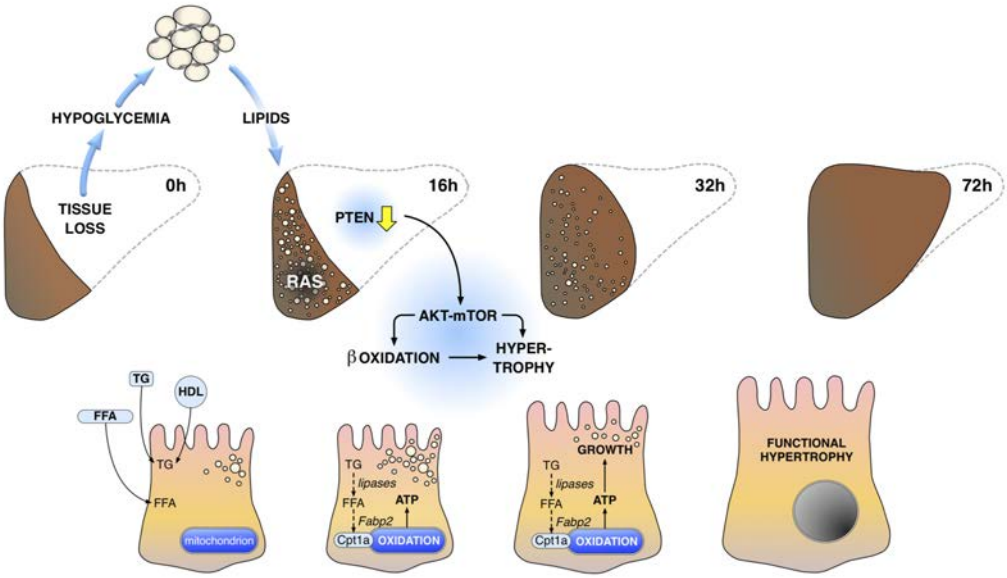

Figure 7. Model for the function of PTEN downregulation after hepatectomy. The hypoglycemia developing with resection triggers the mobilization of adipose stores to redistribute fats from the periphery into the liver. PTEN downregulation occurs with the peak of hepatocellular lipid accumulation. The resulting enhancement of AKT-mTOR signaling promotes β-oxidation and cellular hypertrophy, which is fed through the catabolism of RAS-derived lipids. To facilitate lipid usage, PTEN downregulation enhances the expression of lipases to free fatty acids (FFA) from triglycerides (TG) and increases the levels of molecules needed for the transport of FFAs into mitochondria.

Supplementary Materials

Supplementary methods

Water content

Weighed livers (approx.100mg) were incubated at 100^0C. After 24 hours, dry liver remnant weight was recorded and the ratio (% dry/total weight) was calculated.

Protein content

The DC™ Protein Assay (Biorad, Hercules, CA) was used to quantify protein content (mg/g tissue) of liver samples.

Chemical lipid content

Liver fat was chemically quantified by the Vanillin method according to Van Handel E. (J Am Mosq Control Assoc. 1985;1:302–304)

Supplementary Tables

Table S1. Antibodies for immunoblots and immunohistochemistry

antigene	company/order no.	dilution
Pten	Cell Signaling, 9559S	1:1000 (WB)
pAkt1/2 (Ser473)	Cell Signaling, 9271S	1:1000 (WB)
pAkt (Thr308)	Cell Signaling, 9275S	1:1000 (WB)
tAkt	Cell Signaling, 9272S	1:1000 (WB)
pAkt2 (Ser474)	Cell Signaling, 8599S	1:1000 (WB)
Akt2	Cell Signaling, 3063S	1:1000 (WB)
pS6K	Cell Signaling, 4267S	1:1000 (WB)
S6K	Cell Signaling, 4267S	1:1000 (WB)
p4EBP	Cell Signaling, 2855S	1:1000 (WB)
4EBP	Cell Signaling, 9644S	1:1000 (WB)
Rictor	Cell Signaling, 2114S	1:1000 (WB)
Raptor	Cell Signaling, 2280S	1:1000 (WB)
Gapdh	Cell Signaling, 5174S	1:1000 (WB)
HRP-linked anti-rabbit	Cell Signaling, 7074S	1:2000 (WB)
HRP-linked anti-mouse	Cell Signaling, 7076s	1:2000 (WB)
Srebp1	Novus Biologicals, NB100-2215	1:500 (IHC)
Ki67	Abcam, ab16667	1:200 (IHC)
pH3	Millipore, 06-570	1:500 (IHC)

Table S2. Taqman gene expression assays

gene name	**Applied Biosystems order no.**
Ccna2	Mm00438063_m1
Ccnb2	Mm01171453_m1
Ccnd1	Mm00432359_m1
Ccne1	Mm00432367_m1
Cd36	Mm01135198_m1
Cpt1a	Mm01231183_m1
Fabp2	Mm00433188_m1
Fabp4	Mm00445878_m1
Fasn	Mm00662319_m1
Fgf21	Mm00840165_g1
G6pc	Mm00839363_m1
Hadha	Mm00805228_m1
Hadhb	Mm01217745_m1
Lipe	Mm00495359_m1
LPL	Mm00434764_m1
Pck1	Mm01247058_m1
Plin2	Mm00475794_m1
Ppargc1a	Mm01208835_m1
Pten	Mm00477208_m1
Scd1	Mm00772290_m1
18S *rRNA*	TaqMan control reagents

Supplementary Figures

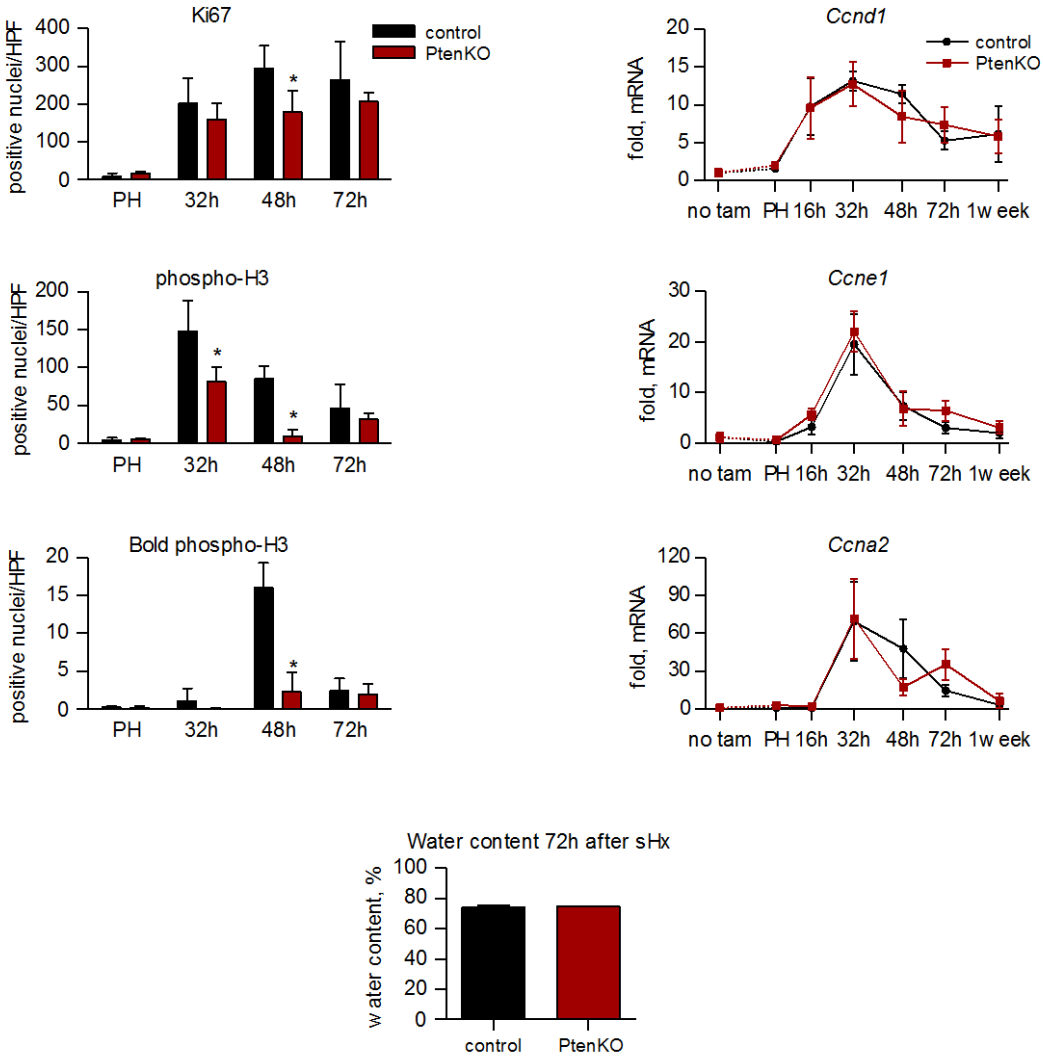

Figure S1. Proliferative parameters in PtenKO and PtenC after PH. A trend towards reduced Ki67 counts was observed in PtenKO from 32h (S phase peak) onwards, suggesting reduced progression to and from the S phase. Total pH3 counts (marking G_2 or M phase cells) were reduced already around the S phase peak, and bold pH3 counts (M phase cells) are reduced around the mitotic peak, again suggesting reduced cell cycle progression. Entry into but reduced progression through the cell cycle is consistent with an elevation of cellular hypertrophy in PtenKO. At 72h after PH (when TG content is close to nil), hepatic water content was similar in both genotypes.

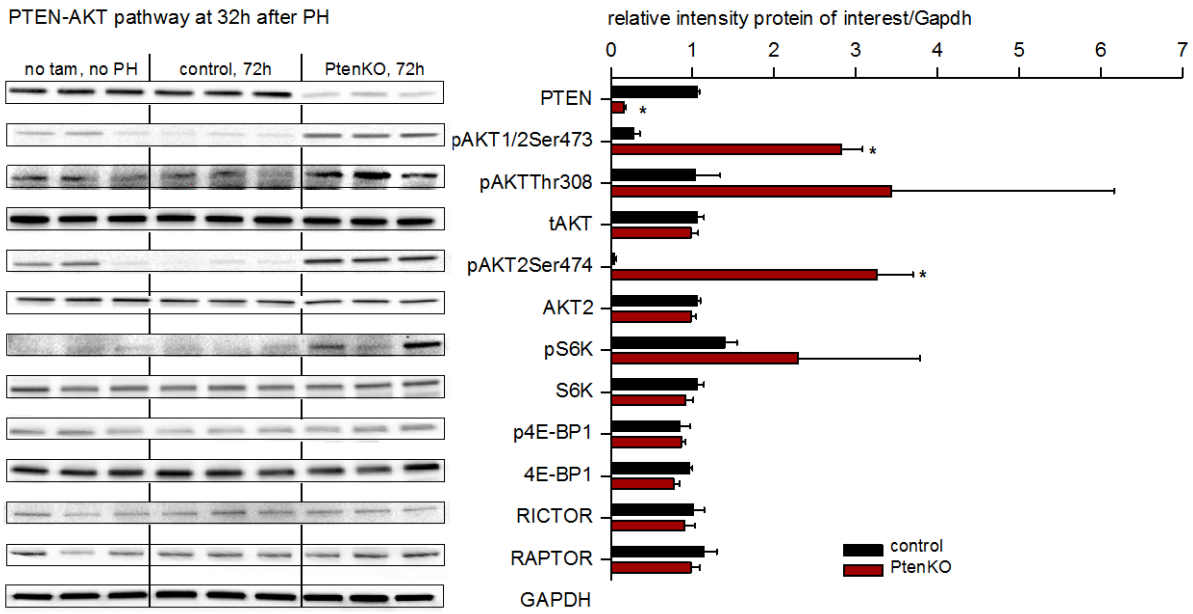

Figure S2. AKT-MTOR signaling in PtenKO/PtenC assessed by immunoblots 32h after PH. PtenKO liver displayed increased phosphorylation of AKT and S6K, indicating upregulated AKT-MTOR signaling at 32h after PH.

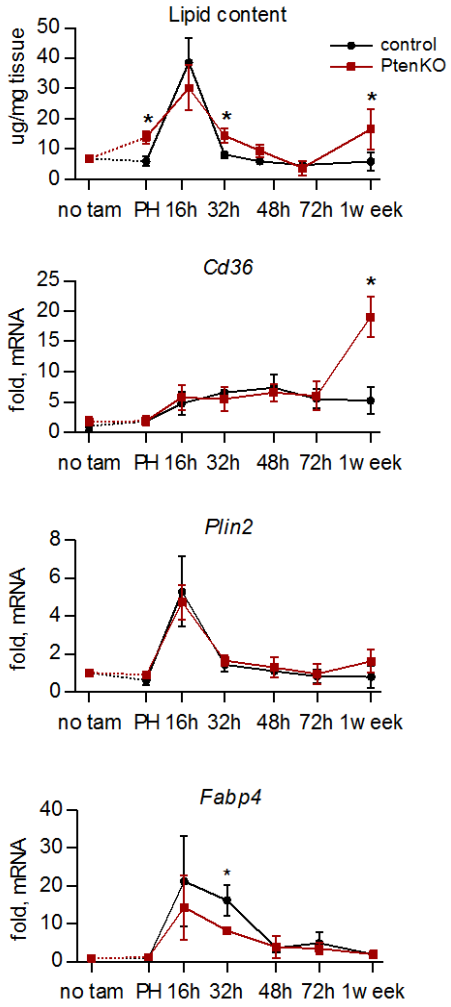

Figure S3. Lipid content, *Cd36* expression and *Plin2* expression in PtenKO and PtenC liver after PH. The assessment of lipid content via the vanillin methodology mirrored the changes in hepatic TGs after PH. *Plin2* and *Cd36* expression were similar in PtenKO and PtenC during the regenerative phase, suggesting PtenKO has little impact on lipid import and vesicle formation. Towards the end of regeneration (1 week) however, lipid content and *Cd36* expression began to rise (with Plin2 displaying an nonsignificant increase) in knockouts, consistent with the phenotype reported for resting PtenKO liver.

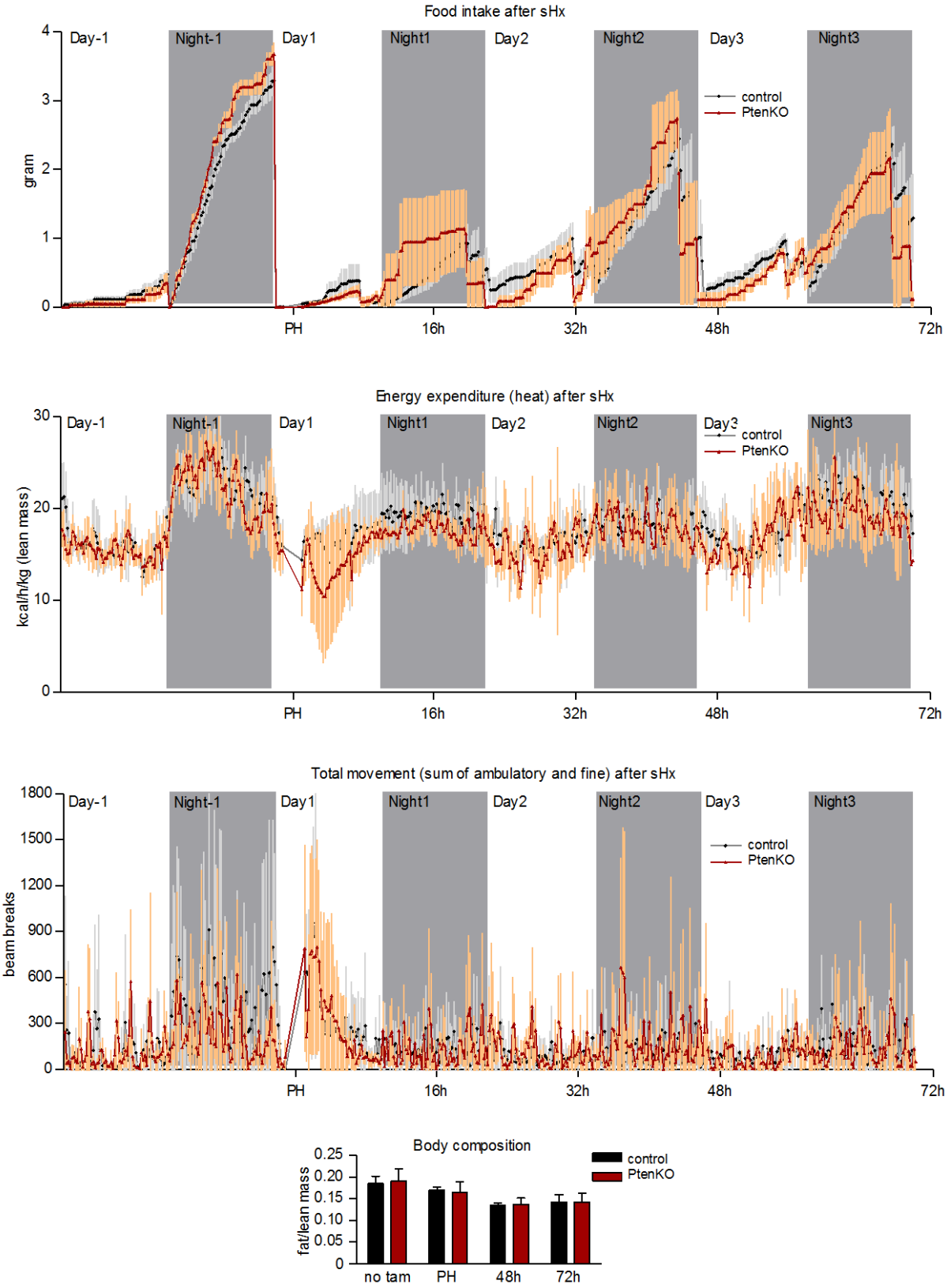

Figure S4. Assessment of food intake, heat production, movement and body composition (fat versus lean mass) in PtenKO and PtenC mice after PH. Data was retrieved from individual mice kept in metabolic cages. No significant differences between the two genotypes were observed.

6. Discussion

The metabolic adaptations that occur immediately after hepatic resection were among the first changes reported for the regenerating liver.

Initially, hypoglycemia and the subsequent accumulation of hepatic lipids were not considered to be of functional relevance, but rather a consequence of deficient liver function. However, growing piles of experimental evidence point to the contrary. Eventually, Rudnick and Davidson summarized these novel insights in a review entitled "Metabolic theory of liver regeneration." In its essence, their message was that the metabolic insufficiencies caused by acute tissue loss have a role in the initiation of liver regeneration [LR], which is equal to that of growth factors, cytokines and other pro-regenerative pathways [89].

During the past few years my main interest was directed towards LR-associated metabolism. For my thesis, I wished to explore (i) how diminished metabolic function of the liver is translated into regenerative signals; and (ii) whether regeneration associated steatosis [RAS] as a metabolic response to hepatic insufficiency is providing energy for tissue recovery.

In brief, my research revealed the following main findings:

- CAR after resection promotes liver function through its metabolic activities (such as bilirubin clearance) and the parallel induction of pro-regenerative molecules, in particular FOXM1. In resection-induced liver failure, CAR activation is deficient and associated with both metabolic and proliferative deficiencies. Reactivation of CAR normalizes all deficiencies in a way dependent on FOXM1, emphasizing that regrowth of liver mass is essential to maintain metabolic capacity.
- After 70% hepatectomy, PTEN is downregulated to promote beta-oxidation of RAS-derived lipids, thereby providing energy for AKT-mTORC1-driven hypertrophy.

Further to the elucidations in our manuscripts, I would like to discuss some more aspects of my work I deem relevant.

6.1 Multiple aspects of CAR action during liver regeneration

The clinical potential of CAR activators was extensively discussed in "Manuscript B". Here I would like to concentrate on the diverse molecular mechanisms which are induced through CAR and result in accelerated LR or even can rescue from the small-for-size syndrome [SFSS].

We demonstrated that FOXM1 is necessary for CAR to exhibit its beneficial effect on LR. It is known that CAR can activate FOXM1 via MYC [75], however it also was shown that FOXM1 on its own is essential for LR itself [32]. Therefore, its knockdown might have obscured FOXM1-independent effects of CAR. Moreover, Foxm1 knockdown did not completely abrogate all effects of CAR activation, providing alternate evidence for FOXM1-independent pathways regulated through CAR during LR.

As already mentioned in the thesis introduction, CAR is known to affect liver growth also through molecules other than MYC/FOXM1 (see Fig. 3 in the introduction). For example, CAR can stimulate the expression of cyclin D directly, thereby promoting also cell cycle entry. Notably, livers following extended hepatectomy exhibit higher expression of cyclin D vs 70% hepactectomy ([63] and data not shown). However, the induction of cyclin D is unlikely to account for the preventive and/or rescuing effects CAR activation had in our SFSS mouse model. The prime defect behind SFSS development is a delay in the progression through the S and M phases; accordingly, we observe reduced cyclin A and B expression after extended hepatectomy relative to standard 70% hepatectomy. Indeed, cyclin D expression is increased after extended hepatectomy, and so are Ki67 counts (i.e. counts of hepatocytes that have entered the cell cycle after resection). It therefore seems that cyclin D is more important for CAR-induced spontaneous hepatomegaly than for the prevention of the SFSS.

On the other hand, CAR has been shown to increase YAP1 activities in the liver [114]. Intriguingly, our preliminary data indicate a deficient induction of YAP1 in the SFSS, which can be normalized through CAR re-activation. Further to this, our data suggest that YAP1 is important for the entry of hepatocytes into the cycle, but also for their further progression to the S phase. We hence believe that the benefits of CAR activation in the SFSS may to some part also rely on the promotion of YAP1 activity after resection.

Finally, CAR can repress P21 not only via FOXM1 or YAP1-dependent mechanisms, but also through the direct inhibition of the Cdkn1a transcriptional promoter FOXO1 [112]. Given that deletion of P21 mitigates most abnormalities associated with the SFSS [63], the direct effects of CAR on P21 are expected to add to the promotion of regeneration in resection-induced liver failure.

6.2 PTEN and steatosis in liver surgery

In the “Manuscript A” covering PTEN's function in LR we discussed potential molecular mechanisms that may mediate the effects elicited through PTEN downregulation. Further to this, I would like to discuss the broader relevance of these findings in clinical settings, particularly with regards to pathological steatosis.

Severe pre-existing steatosis has been identified as a significant risk factor for postoperative complications including liver failure. In both cadaveric and living donor liver transplantation, grafts with severe or moderate steatosis (>30%) are excluded [135]. Likewise, liver resection is considered to be safe only if the degree of steatosis does not exceed 30% [136].

Steatotic liver is highly sensitive towards ischemic injury (often an obligate component of liver surgery) and has a diminished capacity to regenerate. The reasons behind these faults are not completely clarified, but endothelial dysfunction along with altered mitochondrial capacity and oxidative stress are conceivable and accepted causes [137-139]. The options to manage fatty liver disease are rather

limited. Apart from exercise, pre-operative steatosis can be improved within a few weeks via a diet low in fat but rich in protein [140]. Another promising approach is based on omega-3 fatty acids and is currently being trialed in our clinic (NCT01884948). In mouse models, our lab could show that treatment of steatotic liver with omega-3 fatty acids ameliorates hepatic ischemia reperfusion injury and accelerates regeneration via both antisteatotic and steatosis-independent effects [141].

Intriguingly however, others have reported that mild pre-existing steatosis has little or even a beneficial impact on liver regeneration [142, 143]. Such findings suggest that steatosis may be harmful only if above a certain threshold. Additional research is hence needed to define the degree of steatosis, but also the extent of associated changes (e.g. inflammation, lipotoxicity, microcirculation) below which surgery is safe. On the other hand, our findings together with those from several previous studies indicate that the acute development of steatosis after resection is a phase essential for the regenerative process [76, 86, 87]. Our calorimetric measurements demonstrated a clear shift towards the use of lipids as a primary energy source in the initial stage of regeneration. The shift to lipid usage correlated with PTEN downregulation after hepatectomy and was pronounced in our Pten knockout animals. The requirement of fat for liver to regenerate hence explains why mild pre-existing steatosis can promote regeneration. If steatosis however is marked, it starts to impair sinusoidal function and perfusion, which have key roles in the recovery of liver mass.

The loss of PTEN in liver is known to cause steatosis [123, 124]. We induced liver-specific Pten knockout a few days before hepatectomy to avoid the development of significant, pre-existing fatty liver. Despite this short interval, PtenKO mice presented with very mild steatosis already at hepatectomy. Following hepatectomy, PtenKO caused an accelerated liver weight regain. While our experiments using the inhibitor etomoxir showed that the promotion of beta-oxidation through PTEN loss is the main driver behind enhanced regeneration, it is well possible that the mild pre-existing steatosis in PtenKO has aided in this effect.

Along these lines, another noteworthy observation in our PTEN study is the divergent action of this molecule in resting versus regenerating liver. The loss of PTEN in resting liver upregulates lipogenesis, fat import (e.g. Cd36), and induces a mild transdifferentiation of hepatocytes towards adipocytes (as evinced through the upregulation of adipocyte markers such as Plin2 and Fabp4). In contrast, lipogenesis and the expression of Cd36, Plin2 and Fabp4 were similar in PtenKO and PtenC after hepatectomy, indicating little effect of the knockout on these parameters in regenerating liver. These observations imply that the liver regeneration program is highly dominant over the usual processes occurring in resting liver. Thus, the regeneration program appears to adapt PTEN for functions this molecule normally does not exert in resting liver. Indeed - at least to our knowledge - this is the first study that has associated PTEN downregulation with the catabolism of lipids, in stark contrast to many other reports linking PTEN deficiency with the accumulation of lipids [123, 124, 144]. Therefore, the

unique role of PTEN in liver regeneration also highlights the biological plasticity that has evolved to adapt an organism to acute changes.

By fostering the beta-oxidation of RAS-derived lipids, PTEN downregulation promotes hypertrophic liver growth. In PtenKO, the hypertrophic response is enhanced and leads to hepatomegaly. Importantly, this additional liver mass is functional, as demonstrated by the survival raised from 0% in controls to 40% in PtenKO after 91%-hepatectomy (a lethal model of resection-induced liver failure mimicking the small-for-size syndrome). These results suggest that the transient inhibition of PTEN at or after extended resection may be a strategy to improve outcomes of hepatic surgery. Given that cancer is the prime indication for extended resection, the risks associated with PTEN inhibition are obvious. One of the most mutated tumor suppressors in human cancer [145], the loss of PTEN in resting mouse liver leads to the development of steatosis-associated hepatocellular carcinoma over time [124]. However, it is currently unclear whether a short-lived, transient inhibition of PTEN is sufficient to actually promote residual/occult disease in liver. Moreover, inhibition would occur in regenerating liver, where PTEN seems to assume functions different from those in resting liver. Clearly, such risks need a careful assessment, but also have to be outweighed against the potential benefit of PTEN inhibition, i.e. the rescue from sudden death due to postoperative liver failure - still the most frequent cause of death due to liver surgery. Finally, our findings imply that a perioperative supply of lipids together with promoters of beta-oxidation (such as carnitine) might aid the successful completion of regeneration after extended hepatectomy.

Concluding remarks: At the end of the discussion, I would like to draw attention to the similarities that seem to exist for CAR and PTEN in the regenerating liver. PTEN downregulation after hepatectomy promotes liver growth in relation to the suppression of lipogenesis, the oxidation of lipids, and a shift from glucose usage to storage. CAR activation after hepatectomy likewise promotes liver growth; besides its detoxifying and clearance activities, CAR has further been shown to enhance insulin sensitivity and to suppress lipogenesis as well as gluconeogenesis [146, 147]. Whether the latter occurs in a CAR-dependent way also in regenerating liver is not known. Nonetheless, the features associated with PTEN downregulation and CAR activation point to interesting parallels, that is the coupling of growth promoting abilities with the capacity to modulate energy metabolism - features that well might predestine these two molecules to play crucial roles in the regrowth of liver.

Taken together, these two proteins nicely illustrate the reciprocal regulation that occurs between tissue growth and its metabolic performance during liver regeneration: PTEN regulates catabolic metabolism to promote tissue growth, while CAR accelerates liver growth to promote metabolic function.

7. Future directions

YAP1 as a mediator of CAR activities in liver growth

In manuscript B, we demonstrated that the pro-regenerative effects of CAR are implemented through FOXM1. Our results further suggest that other downstream molecules must act to convey the full impact of CAR activation on regenerating liver. Our recent, preliminary data indicate that YAP1 as well is an effector downstream of activated CAR following hepatectomy. Using a siRNA knockdown approach, we are currently working to confirm the role YAP1 appears to have in the CAR-dependent network after resection (Fig. 5). We expect that the establishment of YAP1-regulated effects will help to cement the central position of CAR in the coordination of different stages during liver recovery.

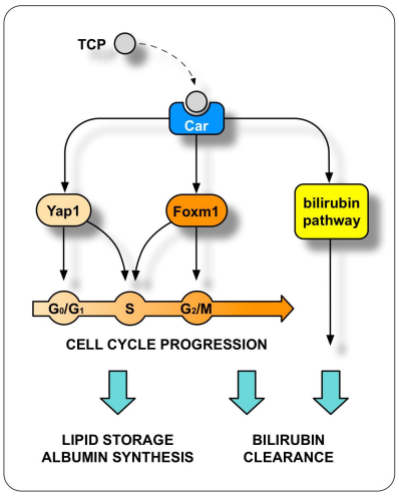

Figure 5. CAR-dependent pathways in the prevention of liver failure following tissue loss. After extended hepatectomy and TCPOBOP (TCP) administration, CAR promotes cell cycle progression through YAP1 and particularly FOXM1. YAP1 mainly stimulates progression through G1 and S, associated with improvements in albumin production and the exaggerated RAS seen in the SFSS. FOXM1 accelerates progression through S and M, improving hypoalbuminemia, persistent steatosis and hyperbilirubinemia. Hyperbilirubinemia likely is reduced also through direct induction of the bilirubin clearance pathway by CAR. (Dr. Humar, unpublished)

Role of PTEN alterations in liver failure

Persistent steatosis and deficient regeneration are two of the characteristic features of SFSS liver. The generic observation that RAS seems to persist after hepatectomy when liver fails to recover suggests that the regenerative delay in the SFSS results in a diminished turnover (e.g. oxidation) of RAS-derived lipids and hence to the persistence of RAS. On the other hand, the tenacity of RAS may itself impair the regenerative capacity of liver.

To address this question, the timing/magnitude of RAS formation and disappearance should be documented at high resolution for both standard and extended resection, and then compared to the onset of regenerative deficits developing with liver failure.

Furthermore, our current data on PTEN downregulation and associated AKT activation after extended hepatectomy are puzzling. Compared to standard hepatectomy, PTEN is equally or even more strongly downregulated, yet activating AKT phosphorylation is reduced. Given that the deletion of PTEN before hepatectomy accelerates regeneration, the prolonged PTEN downregulation seen in the SFSS is

unlikely to lead to a negative feedback that would dampen AKT activities. On the other hand, PTEN is normally downregulated at the peak of RAS; its prolonged downregulation in the SFSS may hence relate to the persistence of RAS (e.g. in the sense that as long there are lipids to be burned, PTEN will remain downregulated to promote their oxidation). Finally, we observed a peculiar downregulation of PTEN one hour (but re-elevation again at 4h and 8h followed by the expected drop at 16h, the RAS peak) after extended but not standard hepatectomy. Again, a fine-tuned time course will be needed to define whether this one-hour-drop is unique to the SFSS or merely shifted in time (e.g. observed 30 min. or 2h after standard hepatectomy).

The information about RAS dynamics and corresponding PTEN changes should provide important information to define potential time windows for a therapeutic exploitation of transient PTEN inhibition against the SFSS.

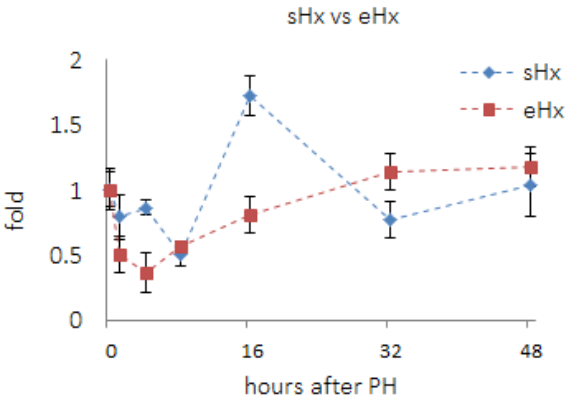

Figure 6. miR-21 expression profile following standard (sHx) and extended (eHx) resection

Molecular mechanisms underlying the function of PTEN in liver regeneration

Upstream of PTEN: What leads to PTEN downregulation after hepatectomy remains unclear. Several reports have proposed regulation of PTEN by microRNAs. For example, the Inhibition of the miR-17~92 cluster in vivo leads to elevated PTEN levels after hepatectomy. However the cluster itself is downregulated after resection and hence cannot explain the reductions in PTEN levels [131]. PTEN downregulation through miR-382 has likewise been proposed, but was only documented in hepatocellular cell lines [133]. Moreover, PTEN downregulation in regenerating liver was associated with miR-21 induction [132]. Although such an association could be documented *in vitro* [148], we could not substantiate a miR-21-PTEN relationship after hepatectomy. We tested this possibility in two systems: (i) we performed hepatectomy in mice with a liver-specific deletion of miR-21, but saw little impact on PTEN levels compared to mice with wildtype miR-21; (ii) we measured miR-21 expression after standard and extended hepatectomy in wildtype mice, but did not observe an inverse correlation between miR-21 and PTEN levels. Indeed, PTEN downregulation was prolonged after extended resection, but miR-21 induction was deficient (Fig. 6), disqualifying this microRNA as a PTEN regulator during LR. However, we plan to follow up the interesting finding of deficient miR-21 expression in the

SFSS, particularly as miR-21 has been assigned a pro-regenerative function in regeneration [149]. The identification of non-coding RNAs that controls Pten expression during regeneration would offer new options (e.g. miRNA-mimics) to modulate PTEN levels after resection.

A close relative of miR-21 - namely miR-22 - has been reported to target Pten and to promote hypertrophy. Moreover, the analysis of constitutive knockout mice has linked miR-22 deficiency to metabolic changes that overlap with the PtenKO phenotype seen in resting liver. To define whether miR-22 is a potential PTEN regulator after resection; its expression should be assessed during the times of PTEN downregulation after both standard and extended hepatectomy. We furthermore plan to assess PTEN levels in liver of hepatocyte-specific miR-22 knockout mice and their controls to obtain functional in vivo evidence for the association between miR-22 and PTEN in regenerating liver.

PTEN-interacting molecules

The AKT-mTOR axis is considered as the main pathway under the control of PTEN. However, PTEN meanwhile is known to exert many other functions and to directly interact with a variety of proteins that may mediate some of its actions. Within the frame of our Sinergia collaboration with the group of Michelangelo Foti (University of Geneve), we are currently defining proteins that might interact with PTEN in regenerating liver. Following their screens in resting, steatotic and cancerous liver, Foti's group has identified several proteins the expression of which is strictly correlated to PTEN alterations Most of these proteins have been assigned tumor suppressor or oncogenic function. Currently, samples from our regenerating livers are being analysed to reveal whether PTEN may interact with these proteins also after tissue loss. Pending on outcomes, functional studies may follow to assign functions to candidate proteins. A desired result would be the dissection LR-associated PTEN functions to specific interaction partners. Such knowledge might enable us to manipulate liver regeneration after extended resection in a more targeted way. For example, one might expect a lowering of tumorigenic risks if cellular growth could be enhanced indirectly, such as the targeted promotion of PTEN-dependent pathways associated with the catabolism of RAS. However, I have learned in my PhD that the road leading to these goals will be long and stony.

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10. Curriculum vitae

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2002 – 2005 Gymnasium No 69, Specialized Chemical-Biological Class, Omsk, Russia

1996 – 2002 General Education School N^{o} 48, Omsk, Russia

Additional training

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2014 Parallels between tissue repair and cancer, ETH, Zurich

2013 Mouse physiology and pathophysiology, UZH, Zurich

2013 Science and grant writing, USZ, Brienz, Switzerland

2013 Scientific writing in sciences and medicine, UZH, Zurich

2012 Course in laboratory animal sciences, FELASA, Zurich

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Publications

1. Tschuor C, Kachaylo E, Perparim L, Raptis DA, Linecker M, Tian Y, Herrmann U, Grabliauskaite K, Weber A, Columbano A, Graf R, Humar B, Clavien PA. Car-driven regeneration protects liver from failure following tissue loss and bears therapeutic potential. J Hepatol. 2016 Jul;65(1):66-74 - with Editorial

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Submitted manuscripts

- Kachaylo E, Tschuor Ch, Calo N, Borgeaud N, Limani P, Piguet AC, Dufour JF, Foti M, Graf R, Clavien PA and Humar B. "Pten downregulation promotes beta-oxidation to fuel hypertrophic liver growth after hepatectomy". Submitted to Gastroenterology, October 2016